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14. ABSTRACT

Recently, various human tumors have been shown to contain cancer stem cells. These cells, although rare, appear to be the only cells that can regenerate tumor and mediate metastasis. Therefore, these rare cancer stem cells may be the only cells that actually matter to a tumor and to therapeutic interventions. Of great interest, these putative cancer stem cells preferentially express markers that are expressed on normal stem/progenitor cells. Prostate cancer (PCa) stem cells have not been definitively identified. Recently, I identified several populations of PCa stem/progenitor cells with different tumorigenicities and primitiveness in human PCa xenografts and cell lines. In this study, we seek to reevaluate the relevancy of my findings in human PCa samples. At present, we have worked with 25 human PCa samples of varying Gleason grades to set up tumorigenicity assays to test tumorigenic potential of CD133 and CD44-positive cells. Importantly, we have performed our homework in terms of fine-tuning conditions to give us prostatospheres and establish primary xenografts in order to carry out our proposed experiments. Preliminary in vitro experiments indicate that CD133 could mark self-renewing PCa stem-like cells in primary PCa samples. We currently have a number of tumorigenicity experiments in incubation and expect to obtain a readout within the next couple of months. We also expect to begin studies on the invasive and metastatic potentials of the populations sorted for our markers of interest in the near future.

15. SUBJECT TERMS
Prostate cancer, stem cells

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INTRODUCTION

In the past few years, a number of solid tumors, including those of the breast (1), brain (2), and colon (3,4), and head and neck (5) have been found contain a spectrum of different cell types, only a rare subset of which are able to initiate tumors. These tumor-initiating cells or cancer stem cells (CSCs) display higher resistance to chemotherapy (6) as well as radiation treatment (7), rendering most current therapeutic regimens targeting cancers ineffective. In order to eradicate tumors and prevent relapse, therapies must be redesigned such that CSCs as well as the remaining bulk of the tumor is eliminated. Prostate cancer (PCa) is a major health threat to American males yet little is known about which cells in a prostate tumor are essential for its maintenance. Our recent work has demonstrated that even in PCa cell lines and xenografts, cells were organized as a hierarchy with differing tumorigenicities. In LAPC9 xenografts, the tumorigenicity is harbored primarily in the Side Population (SP) cells (8). CD44 also appears to identify cells with intermediate cycling properties that were more tumorigenic and metastatic than their isogenic counterparts (9,10). Both populations preferentially express genes that have been linked to self-renewal and maintenance of an undifferentiated state. Our main goal in this DOD Predoctoral Fellowship project is to apply the knowledge obtained from our xenograft studies to freshly isolated primary PCa samples with the goal of identifying and characterizing tumor-initiating stem-like cells in PCa. This knowledge will be useful in the development of diagnostic and prognostic tools and therapeutic regimens to help PCa patients. Three Specific Aims (SAs) have been proposed to achieve the above goal.

1) To identify and characterize putative PCa stem cells in freshly isolated human PCa samples using specific markers

2) To compare the invasive and metastatic potential of the individual purified populations of putative PCa stem/progenitor cells

3) To study the interrelationship among the identified populations of PCa stem/progenitor cells and determine their hierarchical positions in PCa

Since the inception of this fellowship to the present (i.e., Aug. 2007), we have made progress in all 3 SAs, as elaborated below.

BODY

Materials and methods: Main techniques are sketched below. Most of experimental details can be found in references 8 – 10.

a) Primary prostate tumors are obtained immediately after removal from patients and digested for several hours using collagenase type I followed by a short trypsin incubation to break up organoids. A Percoll gradient is then used to remove dead cells and hematopoietic cells and to enrich for parenchymal cells. Stromal cells are eliminated through negative MACS selection using a specific cocktail of antibodies. The remaining cell suspension consists mainly of purified epithelial cells.
b) MACS and FACS are used for sorting of CD44, CD133, and CD57-expressing cells.
c) Clonal and clonogenic assays performed in various anchorage-permissive as well as anchorage-independent conditions.
d) Tumorigenicity assays carried out by injecting purified cells into the subcutaneous site, the subrenal capsule, the dorsal prostate, or the anterior prostate.
e) Immunostaining done to determine colocalization of a panel of markers representing known differentiation states.

RESULTS AND DISCUSSION

I. RESULTS RELATED TO SPECIFIC AIM 1

So far our lab has worked on 40 primary HPCa samples (Appendix I). These samples, which are

Table 1. CD44 and CD133 expression in primary HPCa samples

Sample	Gleason	CD133+ (%)	CD44+ (%)
HPCa4	8	N/D	17.8 (MACS)
HPCa6	7	N/D	4.0 (MACS)
HPCa7	7	N/D	15.0 (MACS)
HPCa8	7	N/D	12.4 (MACS)
HPCa9	6	0.84 (MACS)	N/D
HPCa10	6	N/D	3.3 (MACS)
HPCa12	6	0.61 (MACS)	N/D
HPCa13	6	0.25 (MACS)	N/D
HPCa14	7	N/D	5.63 (IF)
HPCa16	6	0.76 (MACS)	N/D
HPCa17	7	0.1.4 (MACS)	N/D
HPCa18	9	0.94 (MACS)	19.4 (IF)
HPCa25	8	0.73 (MACS)	7.7 (MACS)
LNCaP cells	LN met.	U.D (n=2)/IF	U.D (n=7)/IF+FACS
PC3 cells	BM mets	U.D (n=2)/IF	100 (n=6)/IF+FACS
Du145 cells	brain mets	U.D (n=6)/IF+MACS	28 (n=8)/IF+FACS
Du145 xeno. tumor		U.D (n=3)/FACS	
LAPC4 tumor	LN mets	0.82 (n=8)/F+M	1-5% (n=12)/IF+F
LAPC9 tumor	BM mets	U.D (n=3)/F+M)	14-20 (n=10)/IF+F

Primary human PCa samples were digested into single-cell suspension and used in the analysis of CD44 and/or CD133 expression using either FACS, MACS, or immunofluorescence (IF) staining. Several cultured or xenograft derived PCa cells were similarly analyzed.N/D, not determined; U.D, undetectable.

obtained from radical prostatectomy by either surgery or the Da Vinci procedure and range from ~0.3 – 3 g, are generally shared by several different investigators in the lab for different experimental purposes. Routinely, a portion of the tumor sample is used for establishment of first-generation xenograft tumors in NOD/SCID and for paraffin-embedded sections and cryosections. The majority is used for tissue desegregation and purification of various subpopulations of cells using either FACS (flow activated cell sorting) or MACS (magnetic assisted cell sorting).

Since our experiments with xenograft prostate tumors have revealed that the CD44$^+$ PCa cells are enriched in putative PCa cells (8-10) and others' in vitro work shows that the CD133$^+$ cells may identify stem-like cells in both normal prostate (11) and prostate tumors (12), for the work in this SA, I have focused on these two populations of cells. As illustrated in Table 1 (previous page), using flow (FACS), immunofluorescence (IF), or MACS purification system, I have found both CD44$^+$ and CD133$^+$ tumor cells in primary PCa samples. Interestingly, in this small cohort of fresh clinical samples, the abundance of CD44$^+$ PCa cells seems to correlate with Gleason grade (Table 1). For example, the only Gleason 6 tumor has ~3% of CD44$^+$ cells and the Gleason 7 tumors have ~9.3% CD44$^+$ cells whereas 3 Gleason 8/9 tumors have 17% of CD44$^+$ cells (Table 1). Primary prostate tumors also contain a small population of CD133$^+$ cells, which are more rare than the CD44$^+$ cells (Table 1), consistent with the hypothesis that the CD133$^+$ PCa cells may represent a more primitive subset of tumorigenic cells in the CD44$^+$ PCa cell population (10). Importantly, the CD133$^+$ LAPC-4 cells showed much higher clonogenic potential than the corresponding CD133$^-$ cells (in fact, none of the CD133$^-$ LAPC-4 cells survived the anchorage-independent condition; Fig. 1A). Similarly, the CD133$^+$ HPCa13 cells, when cultured under low O2 (i.e, 1% O2) conditions to minimize the oxidative stress, demonstrated much higher proliferative potential than the corresponding CD133$^-$ cells (Fig. 1B-C). The rarity and the higher proliferative/clonogenic potential of the CD133$^+$ PCa cells are consistent with the proposal that the CD133$^+$ PCa cells may represent the prostate CSCs (10) and also with the fact that this marker has been used to identify normal HSCs and neural SCs as well as glioma and colon CSCs.

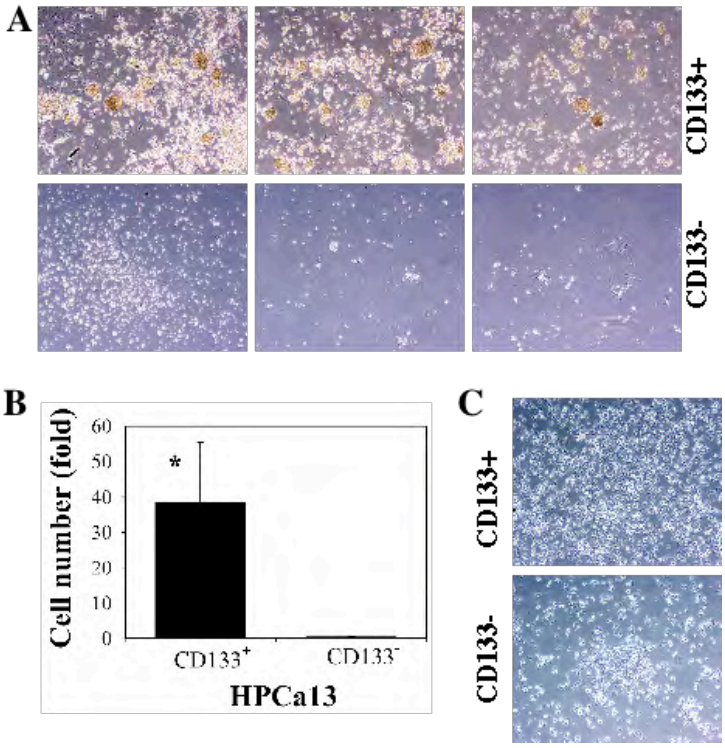

*Figure 1. The CD133$^+$ PCa cells have higher clonogenic (A) and proliferative (B-C) potentials. (**A**) CD133$^+$ and CD133$^-$ cells were acutely purified out from LAPC-4 xenograft tumors and plated (at 10,000 cells/well) on agar for clonogenic assays. Shown are representative images from triplicate wells (x40). (**B-C**) CD133$^+$ and CD133$^-$ cells were purified from HPCa13 primary tumors (Gleason 6). 800 CD133$^+$ and 100,000 CD133$^-$ HPCa13 cells were plated on collagen-coated dished and cultured in 1% O2. Three weeks later, cells were harvested to count cell number (B) and cells were photographed (C; x100).*

Up to this moment, we have implanted the $CD44^+$, $CD133^+$, or $CD44^+CD133^+$, and their corresponding negative cell populations from 5 patient tumors (purified from either primary samples or from the first-generation xenograft tumors) into NOD/SCID mice. We are still monitoring the potential tumor regeneration. We expect that the marker-positive tumor cell populations should possess higher tumor-initiating abilities. If we do observe primary tumor development, we shall compare the histopathology of these 'reconstituted' tumors with that of the primary patient tumor. More importantly, we shall carry out secondary and tertiary tumor development. In the mean time, we are carrying out more tumor experiments with patient samples.

II. RESULTS RELATED TO SPECIFIC AIM 2

Due to the rarity of the putative PCa stem/progenitor cells, we have not yet used patient-derived cells to carry out in vitro invasion assays or in vivo metastasis assays. However, experiments with xenograft human prostate tumors have provided convincing evidence that essentially all metastatic potential resides in the $CD44^+$ PCa cell population (9). We predict to observe similar findings with primary HPCa sample-derived cell populations. When feasible, we shall purify putative CSC-enriched populations from several patient tumors and carry out metastasis-related experiments.

III. RESULTS RELATED TO SPECIFIC AIM 3

We have made significant progress towards accomplishing the goals in this SA, with a recent publication in Cancer Res. (10; Appendix II). Specifically, we found that highly purified (≥98%) $\alpha2\beta1^{+/hi}$ cells from 3 human xenograft tumors, Du145, LAPC-4, and LAPC-9, show higher clonal and clonogenic potential than the $\alpha2\beta1^{-/lo}$ cells in vitro. However, when injected into the NOD/SCID mouse prostate or subcutaneously, the $\alpha2\beta1^{+/hi}$ PCa cells are no more tumorigenic than the $\alpha2\beta1^{-/lo}$ cells. Immunofluorescence studies reveal that CD44 and $\alpha2\beta1$ identify an overlapping and inclusive population of PCa cells in that ~70% of $\alpha2\beta1^{+/hi}$ cells are $CD44^+$ and 20-30% of $CD44^+$ cells are distributed in the $\alpha2\beta1^{-/lo}$ cell population. Subsequently, we sorted out $CD44^+\alpha2\beta1^{+/hi}$, $CD44^+\alpha2\beta1^{-/lo}$, $CD44^-\alpha2\beta1^{+/hi}$, and $CD44^-\alpha2\beta1^{-/lo}$ cells from LAPC9 tumors and carried out tumorigenicity experiments. The results revealed a hierarchy in tumorigenic potential in the order of $CD44^+\alpha2\beta1^{+/hi} \approx CD44^+\alpha2\beta1^{-/lo} > CD44^-\alpha2\beta1^{+/hi} >> CD44^-\alpha2\beta1^{-/lo}$. These observations together provide strong experimental evidence that PCa cells are organized as a hierarchy. Whenever possible, we shall plan similar experiments with several different populations of cells derived from patient tumors.

IV. Major drawbacks and lessons learnt

1) As per the discussion in the original proposal, the gold standard for defining cancer stem cells is those cells that are able to self-renew and harbor the tumorigenicity within the cancer population. In vivo tumorigenicity assays are instrumental in our experiments but unlike the xenograft tumors used in our previous studies that were obtained from metastatic lesions and have adapted to the subcutaneous site for many years, the optimal conditions for growth of primary PCa lesions in immunodeficient mice have not been established. Many reports have been published describing the 'tumor take' in various sites but these studies use the lack of regression of the original implant as a criterion for the 'take' as opposed to actual growth of a tumor. Therefore, we are working from scratch trying to establish the best conditions that would allow a primary tumor to manifest its full tumorigenic (i.e., tumor-regenerating) potential. Using NOD/SCID mice, we have assayed the subcutaneous sites, the dorsal prostate, the anterior prostate, and the subrenal capsule as potential implant/injection sites. Moreover, we have used a diverse combination of support cells to provide a suitable microenvironment for the PCa cells and these include rat urogenital mesenchyme,

carcinoma-associated fibroblasts as well as normal fibroblasts. Unfortunately, the primary xenografts require about 4-5 months post-implantation to form palpable tumors prolonging our experimental incubation time significantly. We have recently obtained preliminary results indicating that the subcutaneous site is the most permissive site for primary tumor development but may not be optimal to assay invasive or metastatic potential. Based on our experiments with xenograft tumors, we hypothesize that the dorsal prostate, being the 'orthotopic' site, may allow the primary tumor to manifest its invasive and metastatic potential. This part of work is being performed by several other co-workers in the lab but the results have direct bearing on the success of this predoctotal fellowship. We are currently summarizing this part of the work for publications.

Our determination of the optimal conditions for the establishment of first and second-generation xenografts will allow us the bypass some of the complications arising from the heterogeneous nature of the samples obtained. Using many tumors from the 'same patient' will reduce the confounding variables in our experimental system, and enable us to perform studies more scientifically. In fact, we have already established primary xenografts from several primary patient samples and are in the process of utilizing them for our assays.

2) A second complication in our system is the heterogeneity of the PCa samples received. PCa by nature has many levels of heterogeneity in that there are often several tumors within a single prostate, each having a different Gleason grade. Furthermore, normal or benign hyperplastic cells often contaminate the sample in varying degrees. A major challenge that lies ahead is to purify marker-positive tumor cells from the normal counterparts because the so-called tumor tissues invariably contain a small percentage of benign tissues. One potential solution is to use macrodissection to specifically pick out tumorous tissues.

KEY RESEARCH ACCOMPLISHMENTS

1) Progress made in optimizing in vitro assays. Conditions for sphere-formation of PCa cells include Matrigel plating as well as culture on Polyhema-coated slides.
2) Progress made in optimizing in vivo assays. The higher Gleason grade tumors appear to form xenografts in the subcutaneous site most efficiently. Previously reported implant sites in the kidney and dorsal prostate rarely allow tumor formation while the anterior prostate appears to be only slightly better. Preliminary results indicate that the rat urogenital mesenchyme may actually promote differentiation of the cancer cells.
3) CD133, a putative marker for normal prostate stem cells (11), is found in all PCa samples examined at a range of 0.25 to 1.4%. CD44, a marker used to identify cancer stem cell populations in many solid tumors (1,5), is expressed in all PCa samples examined at a range of 3.3 to 20%. CD133 expression does not seem to be correlated with Gleason grade but CD44 expression seems to be higher with increased Gleason grade.
4) CD133-expressing cells appear to be significantly enriched for self-renewal as determined by in vitro assays. While $CD133^+$ cells are slow to form sizeable colonies at first, over time, the $CD133^+$ clones become confluent whereas the $CD133^-$ clones continue to die and reduce in number. These experiments are currently being repeated, in addition to in vitro experiments being performed using CD44 as a marker.

5) A number of tumorigenicity experiments using CD133 and CD44-sorted cells are currently in incubation and the results are expected for the next progress report.
6) Most metastatic potential resides in the $CD44^+$ PCa cell population.
7) PCa cells are clearly organized as a tumorigenic hierarchy.

FUTURE PLAN

In the remainder of this fellowship project (~1 year), we expect to have obtained some results of many of tumorigenicity experiments that are currently in process (SA1). We hope to have a clearer picture of the stemness properties of the CD133 and CD44 populations as well as have some of the underlying molecular pathways pinpointed. In addition, we should have an idea of the relationship between cells expressing these markers. Most of the work in SA3 has been accomplished as we have established the basic principle that PCa cells are organized as a tumorigenic hierarchy. Results in SA1 shall provide some clue about tumorigenic hierarchy in primary patient tumors. Furthermore, when patient samples allowed, we shall carry out some prospective experiments to further test this. Finally, we expect to have at least one paper submitted or published on SA2 using both xenograft models and primary patient tumor-derived cells.

REPORTABLE OUTCOMES

Patrawala L, Calhoun-Davis T, Schneider-Broussard R, and Tang DG. Hierarchical organization of prostate cancer cells in xenograft tumors: The $CD44^+\alpha2\beta1^+$ cell population is enriched in tumor-initiating cells. Cancer Res., 67: 6796-6805, 2007.

CONCLUSIONS

Overall good progress has been made towards accomplishing the scientific goals proposed in the original application. The major uncertainty lies in SA1 wherein we have yet to show that, like the situation in xenograft tumors, several populations of marker-positive tumor cells have CSC properties. We expect to see some results to come out soon. Most of the work in SA2 and SA3 have been accomplished except that, when patient samples allow (this generally is the LIMITING factor), we shall carry out some prospective experiments using primary patient-derived tumor cells.

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HPCa primary sample log (updated 080307)

Sample (age)	Date	Race	Pathologist	Diagnosis	Handled by	Usage	Results
HPCa1-T (52)	020405	Hisp	Speights	Garden variety adeno. (acinar)	(DT/BB)	SOI x 4	No T at necropsy
				His. Grade=3; Gleason=7	LP (TC/RBS)	IHC+cryo+tissues frozen	
				Bilaterial; no mets; no inva.		HPCa1-T epi. cells	Frozen vials
						HPCa1-CAFs	Frozen vials
HPCa1-N				Distant from tumor		IHC+cryo+tissues frozen	
(**NHP11**)					LP (TC/RBS)	NHP11-epi. cells	Exp. Frozen vials
						NHP11-fibroblasts	Frozen vials
HPCa2-T (73)	032405	Cauc.	Speights	Adenocarcinoma	(BB)	700k s.c inject. X 2 (032505)	No tumor
				Gleason=6; margin-LN neg.	LP (TC/RBS)	IHC+cryo+tissues frozen	
				multifocal, bilateral		HPCa2-T epi. cells	Frozen vials
						HPCa2-CAFs	Frozen vials
HPCa2-N				Distant from tumor	(DT/BB)	IHC+cryo+tissues frozen	
(**NHP12**)					LP (TC/RBS)	NHP12-epi. cells	Exp. Frozen vials
						NHP12-fibroblasts	Frozen vials
HPCa3-T (73)	070705	Cauc.	Rao	Adenocarcinoma	(BB/JR)	Kid. Cap implan.	No T at necropsy
				Gleason=7; LN neg.	CJ (TC/RBS)	10k (SOI) and 10k+TP (SOI)	No T yet
				multifocal, organ-invasion and		HPCa3-T epi. cells	Frozen vials
				small tumors at left bladder neck		IHC+cryo+tissues frozen	
HPCa3-N				Distant from tumor		NHP13-fibroblasts	N/A
(**NHP13**)					CJ (TC/RBS)	NHP13-epi. cells	Exp. Frozen vials
HPCa4-T (63)	091505	???.	Speights	Gleason=7	(BB/DT)	KC+IHC+cryo	
				tumor extensive; bilateral,	LP (TC/RBS)	CD44+ (67% pure): + CAFs (1:1)	
				extensive perineural invasion		1k x 2; 10kx2; 100kx1→AP (the 10k mice drowned 9/24)	
				through capsule, external margin		CD44- (91% pure): + CAFs (1:1)	
				vascular invasion; nodes nega.		10kx2; 500kx2→AP	
HPCa4-N				Distant from tumor		NHP13-fibroblasts	
(**NHP14**)					LP	CD44+ (31% pure)	
						5k+UGM; 70k+UGM→KC	Harvested 1-13-06
						CD44- (100% pure)	
						70k+UGM→KC	Harvested 1-13-06

HPCa primary sample log

Sample (age)	Date	Race	Pathologist	Diagnosis	Handled by	Usage	Results
HPCa5-T (72)	101005	???.	Speights	Gleason=9	(TC)	IHC+cryo	
				tumor goes thru capsule,	LP (TC/RBS)	ØN digestion	
				but margins neg.		Only 33,000 total cells obtained	
				involves left SV		3k/TR - KC in one mouse	
				bilateral LN neg.		10kx2; 500kx2→AP	
HPCa5-N				Distant from tumor		30k/TR - KC in one mouse	
(**NHP15**)					LP	1) Save one N and one T piece for CJ RT-PCR analyses	
						2) Put several pieces under different culture conditions	
						3) Freeze down several pieces	
HPCa6-T (68)	010306	???.	Dalton	Gleason = 7 (minimal grade 4	TC	IHC+cryo	
				pattern); no margin;	TC	KC/AP (2 mice each)	
				no SV involvement;	LP/CJ	Pieces for RNA and protein	
				Path. Stage=T2a	LP	ØN digestion (collagenase); ~$8x10^6$ cells obtained	
						No Histopaque; CD44 MACs; then TRs done	
HPCa6-N				Distant from tumor		30k/TR - KC in one mouse	
					LP/CJ	1) IHC+cryo	
						2) Pieces for RT-PCR + Western	
						3) Rest frozen down	
HPCa7-T (66 yr)	010606	??.	Speights	Gleason 7 (4+3); bilateral	TC	IHC+cryo	
				extensive, especially on left	TC	KC/AP (1 mouse each)	
				perineural invasion	LP/CJ	Pieces for RNA and protein	
				T. extends into capsule	LP	18h digestion (collagenase); ~$3.5x10^6$ cells obtained	
				w/posi. Margin on left; SV		$1.2x10^6$ cells after Histopaque; CD44 MACs	
				& LN free		CD44+ (180K); CD44- (480K)	
HPCa7-N				Distant from tumor		Clonal+clonogenic+staining on coverslips	
					LP/CJ	1) IHC+cryo	
						2) Pieces for RT-PCR + Western	
						3) Rest frozen down	

HPCa primary sample log

Sample (age)	Date	Race	Pathologist	Diagnosis	Handled by	Usage	Results
HPCa8-T (56 yr)	011106	??.	Dalton (Bob)	Gleason = 6 (3+3)	TC	IHC+cryo	
(Dr. S. Pickett)			0.3 g	PSA=7.7 ng/ml; BPH history	TC	KC/AP (1 mouse each)	
				perineural invasion	LP/CJ	Pieces for RNA and protein	
				T. extends into capsule	LP	16h digestion (collagenase); ~5x10^6 cells obtained	
				w/posi. Margin on left; SV		4.05x10^6 cells after Histopaque; 1.91x10^6 cells after Lin-	
				& LN free		Macs puri.; CD44+ (238K); CD44- (1.5x10^6)	
HPCa8-N						KC + AP implantation (see database)	
				Distant from tumor	LP/CJ	1) IHC+cryo	
						2) Pieces for RT-PCR + Western	
						3) Rest frozen down	
HPCa9-T (9)	012406	??.	Speights	Gleason = 6 (3+3)	TC	IHC+cryo	
(Dr. Waxman)			0.6 g	Gleason 7 on Path. report	TC	KC/AP (1 mouse each)	
				All margins, LN & SV	LP/CJ	Pieces for RNA and protein	
				free of tumor	LP	15h digestion (collagenase); ~7.25x10^6 cells obtained	
						6x10^6 cells after Histopaque; 2.1x10^6 cells after Lin-	
						Macs puri.; CD57+ (250K); CD57-CD44+ (55k)	
						→CD133+ (65K; 10% pure)+CD133- (290K; 100% pure)	
						TR/KC implantation (see database)	
HPCa9-N				Distant from tumor	LP/CJ	1) IHC+cryo	
						2) Pieces for RT-PCR + Western	
						3) Rest frozen down	
HPCa10-T (71)	020806	??.	Dalton (Bob)	Gleason = 6	TC	IHC+cryo	
(Dr. S. Pickett)			2.5 g	PSA=5.9 ng/ml; minmal T. burden	TC	KC/AP (1 mouse each)	
				5% left lateral apex	LP/CJ	Pieces for RNA and protein	
				1% from the right mid gland	LP	15h digestion (collagenase); **rotator stopped mid-way**	
				stage T2 (clinical T1C)		~3x10^6 live cells after Histopaque; 1.4x10^6 cells after Lin-	
				margin and SV neg.		Macs puri.; CD44+ (60K; 80% pure);	
						CD44- (250K; 99% pure);	
HPCa10-N				Distant from tumor	LP/CJ	1) IHC+cryo	
						2) Pieces for RT-PCR + Western	
						3) Rest frozen down	

HPCa primary sample log

Sample (age)	Date	Race	Pathologist	Diagnosis	Handled by	Usage	Results
HPCa11-T (69 yr)	022306	??.	Dalton	Gleason = 8 on biopsy	TC	IHC+cryo	
(Dr. D. Phillips)			(formalinx 20')	Gleason = 7 (4+3) in Path.	TC	s.c (2 pics/mouse x 2; T pellet)	
				both SV with HG infiltrative PCa	LP	*Only 1 x10^6 cells obtained after Histo	
				with extraprosa.+; perineural tumor+		*Plated on 12-well coverslips	
				PSA=4 ng/ml; no BPH history		*200k/AP (x1) and 50K/TR-KC (1x)	
HPCa11-N				Distant from tumor	LP/CJ	1) IHC+cryo	
						2) Pieces for RT-PCR + Western	
						3) Rest frozen down	
HPCa12-T (59 yr)	022806	??.	Dalton (Bob)	Gleason = 6 on biopsy	TC	IHC+cryo	
(Dr. D. Phillips)			??? g	Gleason = 7 (4+3) in Path.	TC	s.c (2 pics/mouse x 2; T pellet)	
				both SV with HG infiltrative PCa	LP	*6 x 10^6 cells after HistøLin-	
				with extraprosa.+; perineural tumor+		*3.5 x 10^6 cells used for direct CD133 MACS	
				PSA=5.7 ng/ml; no BPH history		*CD133+ (21.5k)/CD133- (3.6 x 10^6)→TR/KC	
						*CD133+: 100x10 (3 m); 1kx10 (5 m); 10kx1 (1 m)	
						*CD133-: 1kx8 (4m); 10kx8 (4m); 100kx5 (5m);	
						10^6 x 3 (3 mice)	
HPCa12-N				Distant from tumor	LP/CJ	1) IHC+cryo	
						2) Pieces for RT-PCR + Western	
						3) Rest frozen down	
HPCa13-T (62 yr)	022806	??.	Speights	Gleason = 6 (3+3)	TC	IHC+cryo	
(Dr. P. Reilly)			0.4 g		TC	s.c (2 pics/mouse x 2; T pellet)	
					LP	*1 x 10^6 cells after HistøLin-	
				with extraprosa.+; perineural tumor+		*CD133+ (2500)/CD133- (300,000)	
				PSA=5.7 ng/ml; no BPH history		*Plated on 12-well coverslips	
						*In vitro assays	
HPCa13-N				Distant from tumor	LP/CJ	1) IHC+cryo	
						2) Pieces for RT-PCR + Western	
						3) Rest frozen down	

Sample (age)	Date	Race	Pathologist	Diagnosis	Usage
HPCa14-T (68 yr)	041906	C	Dalton	Gleason = 6-7 on biopsy Gleason = 7 in Path. (prom. Grade 4) PSA=14 ng/ml; no margin	IHC+cryo Both in vitro and in vivo experiments
HPCa14-N				Distant from tumor	1) IHC+cryo 2) Rest frozen down
HPCa15-T (64 yr)	052506	C.	Sang/Pickett	Gleason = 7 on biopsy (2/6 + biopsy) Gleason = 8 in Path. (mainly left prostate; comprising ~30% of tissue)	IHC+cryo Both in vitro and in vivo experiments
HPCa15-N				Distant from tumor	1) IHC+cryo 2) Rest frozen down
HPCa16-T (58 yr)	062906	C.	Dalton	Gleason = 6 on biopsy Gleason = 6 in Path, bilateral ~30% involvement; no M	IHC+cryo Both in vitro and in vivo experiments
HPCa16-N				Distant from tumor	1) IHC+cryo 2) Rest frozen down
HPCa17-T (60 yr)	072606	C.	Dalton	Gleason = 7 on biopsy (28-91% invol.) Gleason = 7 in Path mostly Gleason 4 ~30% involvement; no M	IHC+cryo Both in vitro and in vivo experiments
HPCa17-N				Distant from tumor	1) IHC+cryo 2) Rest frozen down
HPCa18-T (68 yr)	082906	C.	Dalton	Gleason = 9 on biopsy (11/12 cores positive) Gleason = 7 in Path mostly 4) perineural invasion obvious	IHC+cryo Both in vitro and in vivo experiments Tissues implanted; frozen for RNA/pro.
HPCa18-N				Only 1/12 areas is benign	1) IHC+cryo 2) Rest frozen down
HPCa19-T (61 yr)	091406	C.	Fagin/Snider	Gleason = 9 on biopsy Gleason=9 in Path (4+5) Tumor 80-85%; extensive invasion	IHC+cryo Tissues implanted; frozen for RNA/pro. PSA exp. done
HPCa19-N				Benign tissue	1) IHC+cryo 2) Rest frozen down

Sample (age)	Date	Race	Pathologist	Diagnosis	Usage
HPCa20-T (63 yr)	092806	C	Fagin/Eckert	Gleason = 6-8 on biopsy Gleason=9 in Path (4+5)	IHC+cryo Only tumor implantation experiment
HPCa20-N				Distant from tumor	1) IHC+cryo 2) Rest frozen down
HPCa21-T (…yr)	121406	C	Fagin/Snider	Gleason = 6-9 on biopsy Gleason=10 (5+5)	IHC+cryo Only tumor implantation experiment
HPCa21-N				Distant from tumor	1) IHC+cryo 2) Rest frozen down
HPCa22-T (63yr)	011107	C	Fagin/Snider	Gleason = 7 on biopsy Gleason=7 (3+4)	IHC+cryo Tumor implantation experiment+CJ's in vitro
HPCa22-N				Distant from tumor	1) IHC+cryo 2) Rest frozen down
HPCa23-T (72 yr)	013107	C	Fagin/Snider	Gleason = 7 on biopsy (Path. Report does NOT match)	IHC+cryo Tumor implantation experiment+CJ's in vitro *This sample was also sent to Hayward (Feb. 1, 07)
HPCa23-N				Distant from tumor	1) IHC+cryo 2) Rest frozen down
HPCa24-T (62yr)	030107	C	Fagin/Barre	Gleason = 6,7,9 on biopsy Gleason=9 (4+5) on Path. Bilateral, majority of tissue sampled extraprostatic extension: positive	IHC+cryo Tumor implantation using large pieces s.c ONLY
HPCa24-N				Distant from tumor	1) IHC+cryo 2) Rest frozen down
HPCa25-T (58yr)	030807	C	Fagin/Snider	Gleason = 6,7,9 on biopsy Gleason=8 (3+5) on Path. Invasive T: 14/26 slide; 25-30% parenchyma	IHC+cryo Tumor implantation using large pieces s.c ONLY CJ used for in vitro
HPCa25-N				Distant from tumor	1) IHC+cryo 2) Rest frozen down
HPCa26-T (61yr)	040507	C	Fagin Vilmos Thomazy	Gleason = 6,7,8 on biopsy Gleason= 6 on Path. Bilateral, majority of tissue sampled extraprostatic extension: positive	IHC+cryo Tumor implantation using large pieces s.c
HPCa26-N				Distant from tumor	1) IHC+cryo 2) Rest frozen down

Sample (age)	Date	Race	Pathologist	Diagnosis	Usage
HPCa27-T (47 yr)	041907	C	Fagin/Eckert	Gleason = 7-9 on biopsy **Gleason=8** in Path (4+4) Involves ~65% organ Tumor present in all 4 quadrants	IHC+cryo Tumor implants+ in vitro exp.
HPCa27-N				Distant from tumor	1) IHC+cryo 2) Rest frozen down
HPCa28-T (51 yr)	041907	C	Fagin/Farhood	Gleason = 7-9 on biopsy **Gleason=9** in Path (5+4) Acinar-type Pca; + extraprostate ext. Extensive perineural invasion	IHC+cryo Tumor implants+ pilot digest.+cells for CJ
HPCa28-N				Distant from tumor	1) IHC+cryo 2) Rest frozen down
HPCa29-T (68 yr)	050707	C	P. Ruff/Walther	Gleason = 6 on biopsy **Gleason=6** in Path (3+3) Small focus; <10%	IHC+cryo Sofia used for comparative digestion exp.
HPCa29-N				Distant from tumor	1) IHC+cryo 2) Rest frozen down
HPCa30-T (63 yr)	051007	C	Fagin/?	Gleason = 7 on biopsy **Gleason= 7 in path.** Small focus; <10% Extensive perineural invasion	IHC+cryo Sofia practice + Cells for CJ
HPCa30-N				Distant from tumor	1) IHC+cryo 2) Rest frozen down
HPCa31-T (63 yr)	051707	C	Fagin/?	Gleason = 7 on biopsy **Gleason=7** Involves ~25% organ; GS7 in L lateral mid & apex; GS6 in L medial apex	IHC+cryo Sofia sorting: $CD44^{+/-}CD133^{+/-}$ for array
HPCa31-N				Distant from tumor	1) IHC+cryo 2) Rest frozen down

HPCa primary sample log

Sample (age)	Date	Race	Pathologist	Diagnosis	Usage
HPCa32-T (55 yr)	052407	C	Fagin/Eckert	Gleason = 7 on biopsy **Gleason=7 (3+4)** (pT2c pNX pMX G3) Involves ~42% organ; tumor present in all R quadrants except R medial apex Invades into (not thru) capsule	IHC+cryo Tumor implants + in vitro exp. + cytospin slides
HPCa32-N				Distant from tumor	1) IHC+cryo 2) Rest frozen down
HPCa33-T (58 yr)	052407	H	Fagin/Eckert	Gleason = 7 on biopsy **Gleason=7 (3+4)** (pT2c pNX pMX G3 R1) Involves ~50% organ; GS7 in R base & lateral, GS6 in R medial apex & L lateral mid	IHC+cryo Tumor implants + cells for JQ
HPCa33-N				Distant from tumor	1) IHC+cryo 2) Rest frozen down
HPCa34-T (58 yr)	053107	C	Fagin/Eckert	Gleason = 7 on biopsy **Gleason=7** Involves ~67% organ; GS7 in R & L base, R apex & R medial mid; GS6 in R lateral mid	IHC+cryo Tumor implants + in vitro exp.
HPCa34-N				Distant from tumor	1) IHC+cryo 2) Rest frozen down
HPCa35-T (56 yr)	060707	C	Fagin/Eckert	Gleason = 7 on biopsy **Gleason= 8 (4+4);** stage II (pT2c pNX pMX G3) Involves ~15% organ; mainly in left lobe Invades into but not thru capsule	IHC+cryo JQ used for in vitro
HPCa35-N				Distant from tumor	1) IHC+cryo 2) Rest for Mahipal
HPCa36-T (65 yr)	060707	C	Fagin/Barre	Gleason = 7 on biopsy **Gleason=9 (4+5);** pT3b pR1 pN0 PNX Bilateral, involves ~80% organ Invasion of SV and extraprostatic tissues	IHC+cryo Tumor implants + in vitro exp.

HPCa primary sample log

Sample (age)	Date	Race	Pathologist	Diagnosis	Usage
HPCa36-N				Distant from tumor	1) IHC+cryo 2) Rest for Mahipal
HPCa37-T (59 yr)	061407	C	Fagin/Barre	Gleason = 8 (4+4) on biopsy **Gleason=9 (4+5)** on Path. (pT3b R1 pNX pMX) Involves 95% tissue PSA = 21.6; extensive extraprosta. Invasion SV positive	IHC+cryo Purified cells for CJ (KC nanog exp.) Jichao for PSA+/- sorting
HPCa37-N				Distant from tumor	1) IHC+cryo
HPCa38-T (67 yr)	061407	C	Fagin/Barre	Gleason = 8 (4+4) on biopsy **Gleason=9 (4+5)** on Path. (pT3b RpNX pMX) Involves 40% tissue; invades extraprosta. PSA = 7.9; tight SV involved	IHC+cryo Sofia used to purify cells for array
HPCa38-N				Distant from tumor	1) IHC+cryo
HPCa39-T (58 yr)	062807	C	Fagin/Vendrell	Gleason = 7 (4+3) on biopsy **Gleason=7(3+4)** on Path. (pT3a NX MX G3-4) Involves 50% tissue and both lobes	~2g tumor; IHC+cryo; s.c implant. 3 IR NOD/SCID CD44CD133 cells (SH)+in vitro exp. ~1.8 g tumor for Jichao: PSA experiment
HPCa39-N				Distant from tumor	1) IHC+cryo
HPCa40-T (58 yr)	062807	C	Fagin/Barre	Gleason = 7 (4+3) on biopsy **Gleason=7(3+4)** on Path. (pT2a NX MX) Involves <50% of the left lobe No extraprostate invasion	~1.5g tumor; IHC+cryo; Implant 4. IR NOD/SCID CD44CD133 cells (SH)→no in vitro exp.
HPCa40-N				Distant from tumor	1) IHC+cryo

Hierarchical Organization of Prostate Cancer Cells in Xenograft Tumors: The CD44$^+\alpha2\beta1^+$ Cell Population Is Enriched in Tumor-Initiating Cells

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Abstract

Prostate cancer cells are heterogeneous in their tumorigenicity. For example, the side population cells isolated from LAPC9 xenografts are 100 to 1,000 times more tumorigenic than the corresponding non–side population cells. Highly purified CD44$^+$ prostate cancer cells from several xenografts are also enriched in prostate cancer stem/progenitor cells. Because the CD44$^+$ prostate cancer cell population is still heterogeneous, we wonder whether we could further enrich for tumorigenic prostate cancer cells in this population using other markers. Integrin $\alpha2\beta1$ has been proposed to mark a population of normal human prostate stem cells. Therefore, we first asked whether the $\alpha2\beta1^{+/hi}$ cells in prostate tumors might also represent prostate cancer stem cells. Highly purified ($\geq$98%) $\alpha2\beta1^{+/hi}$ cells from three human xenograft tumors, Du145, LAPC4, and LAPC9, show higher clonal and clonogenic potential than the $\alpha2\beta1^{-/lo}$ cells *in vitro*. However, when injected into the nonobese diabetic/severe combined immunodeficient (NOD/SCID) mouse prostate or s.c., the $\alpha2\beta1^{+/hi}$ prostate cancer cells are no more tumorigenic than the $\alpha2\beta1^{-/lo}$ cells. Immunofluorescence studies reveal that CD44 and $\alpha2\beta1$ identify an overlapping and inclusive population of prostate cancer cells in that $\sim$70% of $\alpha2\beta1^{+/hi}$ cells are CD44$^+$ and 20% to 30% of CD44$^+$ cells are distributed in the $\alpha2\beta1^{-/lo}$ cell population. Subsequently, we sorted out CD44$^+\alpha2\beta1^{+/hi}$, CD44$^+\alpha2\beta1^{-/lo}$, CD44$^-\alpha2\beta1^{+/hi}$, and CD44$^-\alpha2\beta1^{-/lo}$ cells from LAPC9 tumors and carried out tumorigenicity experiments. The results revealed a hierarchy in tumorigenic potential in the order of CD44$^+\alpha2\beta1^{+/hi}$ $\approx$ CD44$^+\alpha2\beta1^{-/lo}$ > CD44$^-\alpha2\beta1^{+/hi}$ $\gg$ CD44$^-\alpha2\beta1^{-/lo}$. These observations together suggest that prostate cancer cells are organized as a hierarchy. [Cancer Res 2007;67(14):6796–805]

Introduction

The cancer stem cell model posits that not all cells in a tumor are equal, and that tumor-initiating cells are a rare subset with a distinct phenotype (1, 2). This hierarchical model helps explain why most tumors are heterogeneous although they have a clonal origin; why it is often difficult to establish a permanent cell line from primary tumors; and why it takes tens of thousands of cancer cells to reestablish a tumor. Indeed, cancer stem cells have been shown to be the driving force behind tumor formation in several types of cancers, including those of the blood, breast, brain, and colon (3–7). Cancer stem cells are generally thought of as self-renewing cells that are able to reinitiate a tumor for several generations in NOD/SCID mice and can give rise to a spectrum of differentiated cells (1, 2, 8, 9). Cancer stem cells, like normal stem cells, are also more likely to express antiapoptotic and drug-resistance genes, making them impervious to most anticancer therapeutics (1, 8). To completely eradicate a tumor and prevent recurrence, it is imperative that cancer stem cells be specifically targeted.

Prostate cancer is the second most common type of cancer afflicting American males, and yet, little is known about which cell types within the prostate are the targets of tumorigenic transformation (reviewed in ref. 9). The normal human prostate contains two major epithelial cell types: luminal and basal cells. The luminal cells express cytokeratins 8 and 18, androgen receptor, prostate-specific antigen, prostatic acid phosphatase, and 15-lipoxygenase 2 (9–11), whereas basal cells express cytokeratin 5, CD44 (12), Bcl-2 (13), p63 (14), telomerase (15, 16), and glutathione *S*-transferase π (17) and display $\sim$75% of mitotic activity in the prostate (18). The human prostate epithelium has the ability to generate gland-like structures when combined with rat urogenital mesenchyme and implanted into the renal capsule (19), suggesting the presence of stem cells. Strong experimental evidence exists that putative human prostate stem cells might localize in the basal cell layer (9). Several candidate populations of prostate stem/progenitors cells have been reported, including those expressing CD44, $\alpha2\beta1$, or CD133 (20, 21). For example, the $\alpha2\beta1^{hi}$ cells comprise $\sim$1% to 15% of the CD44$^+$ basal cell population and seem to possess higher *in vitro* colony-forming efficiency as well as an ability to generate prostate-like acini when engrafted with stromal cells into the flanks of nude mice (20). Further characterization reveals that this proliferation and developmental potential seems to be harbored preferentially by CD133-expressing cells within the CD44$^+\alpha2\beta1^{hi}$ population (21). Our recent work also shows that primary prostate epithelial isolates contain cells that possess tremendous proliferative potential and the ability to "transdifferentiate" into other cell types (9). As the prostate joins the growing list of organs that are found to contain adult stem cells, it seems impossible to ignore the likelihood that prostate cancer development might involve these cells or their immediate progeny. In fact, it has been recently reported that stem-like cells in patient prostate tumors can be identified using the putative normal stem cell phenotype (i.e., CD44$^+\alpha2\beta1^{hi}$CD133$^+$; ref. 22). Not only is this population rare but it also shows the highest colony-forming efficiency and the capacity to differentiate into several cell types. Unfortunately, the ability of these cells to reinitiate serially transplantable tumors, which is the gold standard to define cancer stem cells (8, 9), was not shown (22).

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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We have recently shown that cells in well-established prostate cancer xenografts also seem to be organized as a hierarchy with distinct subsets of cells that preferentially harbor tumorigenicity. For instance, the side population isolated from LAPC9 xenograft tumors is ~1,000 times more tumorigenic than the corresponding non–side population cells, suggesting that the side population is enriched in prostate cancer stem/progenitor cells (23). In addition, highly purified $CD44^+$ cells are also enriched in tumorigenic and metastatic prostate cancer stem/progenitor cells (24). In this study, we continue to use these xenograft models in an attempt to further dissect out tumorigenic prostate cancer stem/progenitor subpopulations. The results reveal that prostate cancer cells show a hierarchy in their tumorigenic potential based on their CD44 and α2β1 expression profiles.

Materials and Methods

Cells, reagents, and animals. LNCaP, Du145, PC3, and PPC-1 prostate cancer cell lines were obtained from American Type Culture Collection and cultured in RPMI containing 7% heat-inactivated fetal bovine serum (FBS). Xenograft human prostate tumors LAPC4 and LAPC9 were obtained from Dr. C. Sawyers (Department of Medicine, Jonsson Comprehensive Cancer Center, David Geffen School of Medicine, University of California at Los Angeles, Los Angeles, CA) and maintained in NOD/SCID mice (24). Du145 xenograft tumors were established using early-passage cells and maintained in NOD/SCID mice (23, 24). NOD/SCID mice were obtained from The Jackson Laboratory or bred in our own animal facility and maintained in standard conditions according to the institutional guidelines. Antibodies used include α2β1 [monoclonal antibody (mAb), Chemicon], CD44 (mAb, BD PharMingen), and ABCG2 (mAb, Chemicon). The mouse phycoerythrin-conjugated anti-human α2β1 mAb was obtained from Chemicon and the FITC-conjugated anti-CD44 antibody from BD PharMingen. The isotype control antibody and FITC-, phycoerythrin-, or AlexaFluor-conjugated secondary antibodies were from Chemicon.

Indirect immunofluorescence and flow cytometric analysis and sorting of $\alpha2\beta1^{+/hi}$ and $\alpha2\beta1^{-/lo}$ cells. Fluorescence microscopy was carried out as previously described (23, 24). For flow cytometry, cells were stained live in the staining solution containing 2% FBS and phycoerythrin-conjugated anti-α2β1 mAb either alone or in combination with FITC-conjugated anti-CD44 antibody (15 min at 4°C). Samples were analyzed on a Coulter Epics Elite flow cytometer and $\sim 5 \times 10^6$ to 10×10^6 cells were typically sorted. Cell debris and clumps were electronically gated out. For the positive population, only the top 10% most brightly stained cells were selected. For the negative population, only the bottom 10% most dimly stained cells were selected. The purities of the sorted populations, as determined by both post-sorting flow analyses as well as restaining followed by fluorescence microscopy analyses, were generally ≥98%.

Clonal analysis and clonogenic assays. *In vitro* colony-forming and clonogenic assays were done as described before (23, 24). Briefly, tumor cells were plated at clonal density (i.e., 100–500 per well in a six-well tissue culture dish). Holoclones with >50 cells were counted at the end of 1 week. For clonogenic assays, cells were plated at 1,000 per well in six-well culture dishes coated with a thin layer of 1% solidified agar. Spheres or spheroids (i.e., colonies) that arose within 1 to 2 weeks were presented as clonogenicity (i.e., percent of the spheres/1,000 cells initially plated). Triplicate samples were run for each cell type and at least two individuals scored the clones and spheres separately in a blind fashion.

Xenograft tumor processing and *in vivo* tumorigenicity experiments. Basic procedures have previously been described (23, 24). In brief, xenograft prostate tumors (Du145, LAPC4, and LAPC9) were minced into ~1-mm^3 pieces in Iscove's modified Dulbecco's medium supplemented with 20% FBS. Tumor tissues were incubated with 1× Accumax (1,200–2,000 units/mL proteolytic activity containing collagenase and DNase; Innovative Cell Technologies, Inc.) at 10 mL/g of tissue in Dulbecco's PBS for 30 min at room temperature under rotating conditions. Single-cell suspension was obtained by filtering the supernatant through a 40-μm cell strainer and cell suspension was then gently loaded onto a layer of Histopaque-1077 gradient and then centrifuged at 400 × *g* for 30 min at room temperature. RBC, dead cells, and debris were removed from the bottom of the tube and live nucleated epithelial cells collected at the interface. The resultant cell mixture was depleted of lineage-positive host cells using the *MACS* Lineage Cell Depletion kit (Miltenyi Biotec). To that end, cells were first incubated (10 min at 4°C) in the staining solution [PBS (pH 7.2), 0.5% FBS, 0.5 μg/mL insulin] containing biotinylated antibodies against a panel of lineage antigens (CD5, CD45R, CD11b, anti–Ly-6G, 7-4, and Ter-119). Cells were then incubated with the anti-biotin microbeads (15 min at 4°C) and the Lin^- cells were eluted using the mass spectrometry columns. The purified human epithelial tumor cells were used in various experiments.

For tumor experiments, cells in 40-μL solution consisting of 50% each medium and Matrigel were injected either s.c. or into the dorsal prostate of NOD/SCID mice (~8 weeks old). In some experiments, testosterone pellets ~0.2 cm in diameter (we used testosterone propionate powder purchased from Sigma to make pellets in our laboratory) were implanted dorsally under the skin of male NOD/SCID mice. Primary tumor sizes were measured with a caliper on a weekly basis. Tumorigenicity was measured mainly by tumor incidence (i.e., the number of tumors/number of injections) and latency (i.e., time from injection to detection of palpable tumors). For the double-sort experiments, all tumor-bearing animals were terminated at the same time when tumor burden became obvious for any one animal in one group. Animals were sacrificed and primary tumors dissected out, and tumor weights were determined. Animals with no sign of tumor burden were also examined on necroscopy to confirm that there was no tumor development.

Results

α2β1 expression correlates with malignancy in prostate cancer cell lines and xenografts. α2β1, an integrin expressed in the human prostate (25) and mediating adhesion to collagen I/IV and laminin I (26), has been proposed to identify normal human prostate stem cells (21). To determine whether prostate cancer cells expressing high levels of α2β1 (which are called $\alpha2\beta1^{+/hi}$ or simply $\alpha2\beta1^+$ in this study) in prostate tumors might also represent prostate cancer stem/progenitor cells, we examined its expression in the commonly used prostate cancer cell lines LNCaP, Du145, PC3, and PPC-1 (27), as well as in the LAPC4 and LAPC9 xenograft tumors (10), using a mAb against the α2 subunit. As shown in Fig. 1, LNCaP cells, which are the least aggressive (27), did not show detectable α2β1 expression revealed by immunofluorescence staining. By contrast, PC3 and its derivative PPC-1 cells, which were the most malignant (27), showed 100% expression (Fig. 1). The three prostate cancer cell types that possess intermediate malignancy (i.e., Du145, LAPC4, and LAPC9) showed intermediate levels (i.e., ~1–10%) of α2β1 expression (Fig. 1). Importantly, α2β1 was also expressed only in a subset of cells in these three xenograft tumors (Figs. 1*B* and 2*A*). Flow cytometry analysis of multiple xenograft tumors indicated that the LAPC9, LAPC4, and Du145 tumors expressed, on average, 2.4% (n = 9), 0.4% (n = 5), and 9.8% (n = 4), respectively, of the $\alpha2\beta1^{+/hi}$ cells (Supplementary Table S1; Fig. 2*A*). Note that the percentages of the $\alpha2\beta1^{+/hi}$ cells detected by flow cytometry were generally slightly higher than those detected by immunofluorescent staining. These results, taken together, suggest that the abundance of $\alpha2\beta1^+$ cells in prostate cancer cell cultures and xenografts is correlated with tumor cell malignancy.

$\alpha2\beta1^{+/hi}$ cells possess higher clonal and clonogenic potentials *in vitro* compared with the isogenic $\alpha2\beta1^{-/lo}$ cells. To determine whether the $\alpha2\beta1^{+/hi}$ prostate cancer cells are intrinsically different from the isogenic $\alpha2\beta1^{-/lo}$ (or simply $\alpha2\beta1^-$) prostate cancer cells, we used flow cytometry to purify

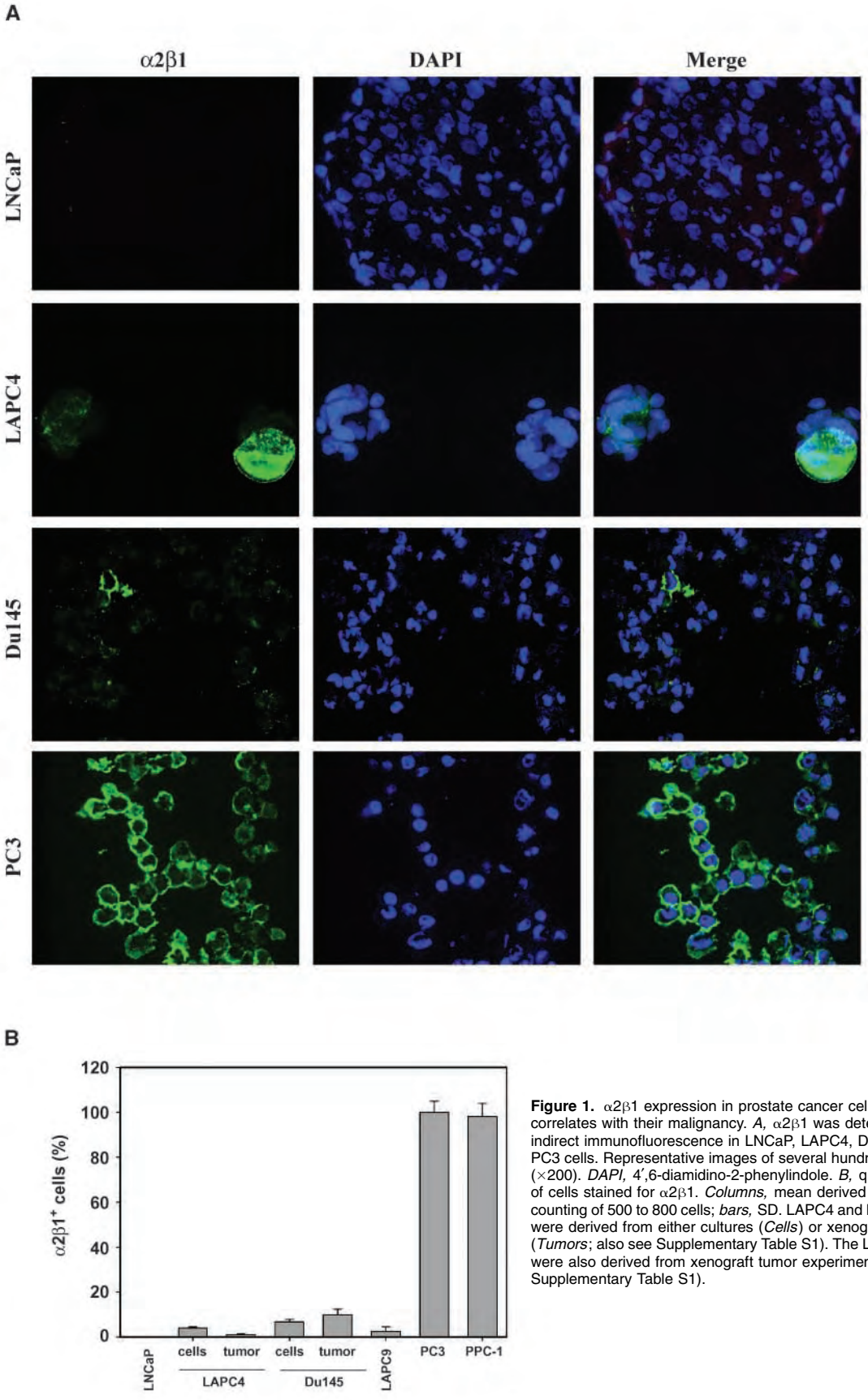

Figure 1. α2β1 expression in prostate cancer cells positively correlates with their malignancy. *A,* α2β1 was detected using indirect immunofluorescence in LNCaP, LAPC4, Du145, and PC3 cells. Representative images of several hundreds analyzed (×200). *DAPI,* 4′,6-diamidino-2-phenylindole. *B,* quantification of cells stained for α2β1. *Columns,* mean derived from a counting of 500 to 800 cells; *bars,* SD. LAPC4 and Du145 results were derived from either cultures (*Cells*) or xenograft tumors (*Tumors*; also see Supplementary Table S1). The LAPC9 results were also derived from xenograft tumor experiments (see Supplementary Table S1).

these two populations of cells from Du145 xenograft tumors. As in our experiments with ABCG2 (23) and CD44 (24), the purities of the $\alpha2\beta1^{+/hi}$ and $\alpha2\beta1^{-/lo}$ cell populations were ≥98% and ~100%, respectively, as revealed by post-sort flow analysis and/or immunostaining (data not shown). When compared for their cloning (i.e., the ability to establish a holoclone; ref. 9) and sphere-forming abilities, the $\alpha2\beta1^{+/hi}$ cells showed significantly higher colony-forming efficiency (Fig. 2*B*) and formed bigger clones (Fig. 2*C*) compared with the isogenic $\alpha2\beta1^{-/lo}$ cell population. The difference observed was not due to variations in adhesion capacity between the two subsets because even in the negative population, a roughly equal number of cells were observed to attach to the dish on initial plating. However, many of the $\alpha2\beta1^{-/lo}$ Du145 cells formed abortive clones or no clones at all. Additionally, when plated at low densities in anchorage-independent conditions (i.e., on soft agar-coated dishes), the $\alpha2\beta1^{+/hi}$ Du145 cells had a much higher sphere-initiating capacity than the corresponding negative population (Fig. 2*D*). The results in Fig. 2 are consistent with the possibility that the $\alpha2\beta1^{+/hi}$ prostate cancer cell population might be enriched in prostate cancer stem/progenitor cells.

$\alpha2\beta1^{+/hi}$ are no more tumorigenic than the corresponding $\alpha2\beta1^{-/lo}$ cells. The gold standard in testing putative cancer stem cells is whether the candidate population of cells can preferentially initiate tumor development in recipient animals (8, 9). Therefore, we carried out surgical orthotopic implantation experiments by injecting varying numbers of acutely purified $\alpha2\beta1^{+/hi}$ and $\alpha2\beta1^{-/lo}$ Du145 cells into the dorsal prostates of NOD/SCID mice. The dorsal prostate has been widely used as the "orthotopic" implantation site for human prostate cancer (28). As shown in Table 1, unsorted Du145 cells, when injected into the dorsal prostate, showed a cell number–dependent increase in tumorigenicity. In general, 100,000 Du145 cells had to be injected to initiate tumor development, and 0.5×10^6 to 2.0×10^6 cells were required to manifest significant tumorigenicity (Table 1). When freshly purified $\alpha2\beta1^{+/hi}$ and $\alpha2\beta1^{-/lo}$ Du145 cells were compared for their tumorigenicities, surprisingly, we did not observe any difference (Table 1). Due to the low percentage of $\alpha2\beta1^{+/hi}$ cells in Du145 tumors (Fig. 1), we were not able to inject more than 10,000 $\alpha2\beta1^{+/hi}$ cells for comparative purposes. One would expect 10,000 $\alpha2\beta1^{+/hi}$ cells to be sufficient for tumor-initiation if these cells were truly primitive with respect to their tumorigenicity; however, this was not the case. The $\alpha2\beta1^{+/hi}$ cells were not more tumorigenic than the same number of unsorted or $\alpha2\beta1^{-/lo}$ cells. In fact, the $\alpha2\beta1^{-/lo}$ population seemed to be slightly enriched in tumorigenic cells such that at 100,000 cells injected, we observed 100% tumor development compared with 25% tumor development with 100,000 unsorted cells (Table 1). The tumor latency was also shorter in the $\alpha2\beta1^{-/lo}$ group than in the unsorted group (Table 1).

We repeated the experiment using LAPC4 and LAPC9 xenograft tumors and obtained similar results (Table 1). The unsorted LAPC4 cells purified from xenograft tumors exhibited tumor rate and latency that were similar to Du145 cells in that 100,000 cells had to be injected into the dorsal prostate to observe any tumor

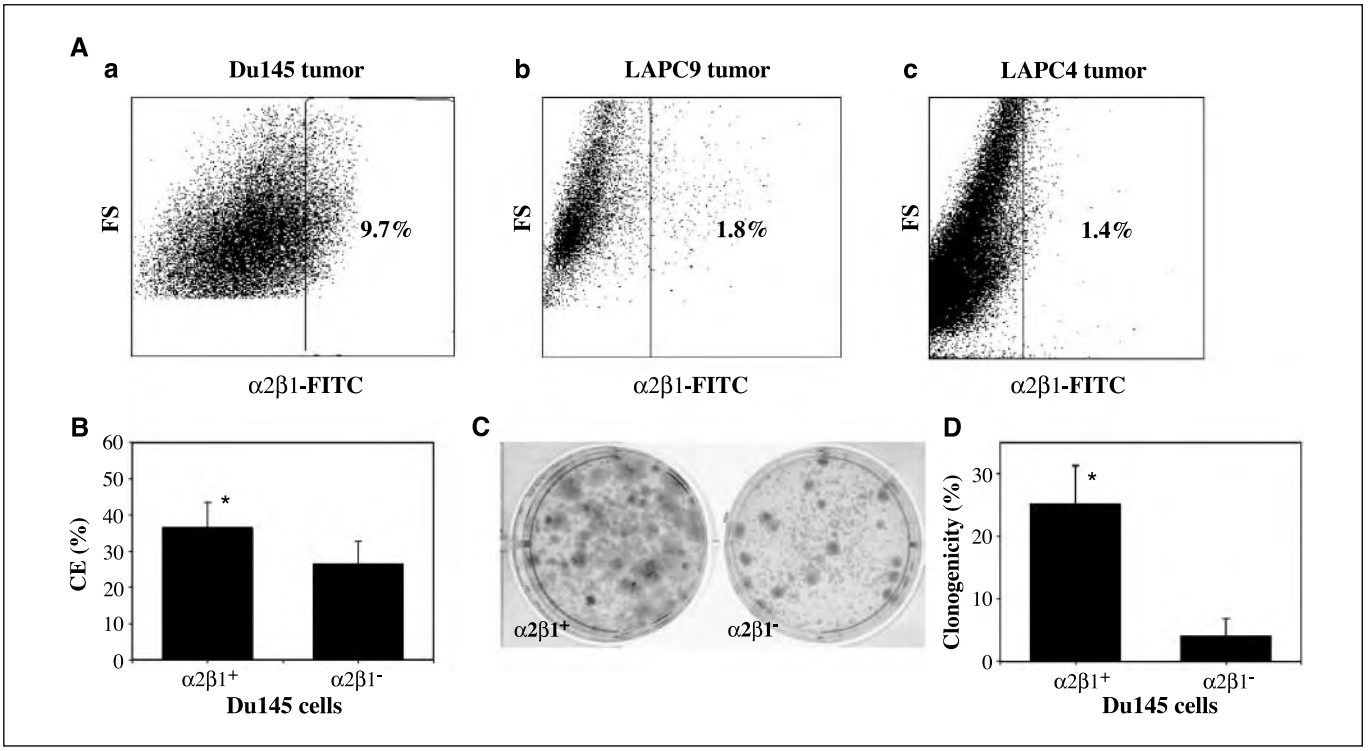

Figure 2. $\alpha2\beta1^{+}$ prostate cancer cells possess higher clonal and clonogenic potential compared with $\alpha2\beta1^{-}$ cells. *A,* flow cytometry analysis of $\alpha2\beta1$ expression in xenograft human prostate tumors. Tumor cells purified from Du145 (*a*), LAPC9 (*b*), or LAPC4 (*c*) were stained live with FITC-conjugated anti-$\alpha2\beta1$ antibody and analyzed on a Coulters Epics Elite flow cytometer. The percentages of $\alpha2\beta1^{hi/+}$ cells are indicated. *B,* Du145 cells were sorted by FACS for $\alpha2\beta1^{hi}$ (i.e., $\alpha2\beta1^{+}$) or $\alpha2\beta1^{lo}$ (i.e., $\alpha2\beta1^{-}$) cells (99% purity), and plated at clonal density (100 per well in a six-well dish) in triplicate. Seven days after plating, clones (with cell number >50) were counted and results were presented as percent cloning efficiency. *Columns,* mean from three independent experiments; *bars,* SE. *, $P = 0.038$, compared with the $\alpha2\beta1^{-}$ group (Student's *t* test). *C,* representative images of clonal analyses (×10). *D,* purified $\alpha2\beta1^{+}$ and $\alpha2\beta1^{-}$ Du145 cells were plated in triplicate at 1,000 per well in a six-well plate coated with soft agar for clonogenicity assays. Spheres were counted 2 wk after plating. *Columns,* mean from three independent experiments; *bars,* SE. *, $P < 0.001$, compared with the $\alpha2\beta1^{-}$ group (Student's *t* test).

Table 1. Tumorigenicity of $\alpha2\beta1^{+/hi}$ and $\alpha2\beta1^{-/lo}$ prostate cancer cells injected into the dorsal prostate

Cell type	No. cells injected	Tumor incidence*	Latency (d) †
Du145, unsorted	1,000	0/4	
	10,000	0/4	
	100,000	1/4	103
	500,000	3/5	53–59 (53)
	2,000,000	3/3	39–74 (46)
Du145-$\alpha2\beta1^{+/hi}$	1,000	0/4	
	10,000	0/8	
Du145-$\alpha2\beta1^{-/lo}$	1,000	0/4	
	10,000	0/4	
	100,000	4/4	53–93 (72)
	500,000	1/1	48
LAPC4, unsorted	100	0/4	
	1,000	0/4	
	10,000	0/4	
	100,000	1/4	103
	500,000	4/5	43–69 (46)
LAPC4-$\alpha2\beta1^{+/hi}$	1,000	0/4	
	10,000	0/2	
LAPC4-$\alpha2\beta1^{-/lo}$	1,000	0/4	
	10,000	1/4	102
	100,000	5/6	53–97 (62)
LAPC9, unsorted	100	0/3	
	1,000	0/9	
	10,000	4/8	46–75 (53)
	100,000	6/9	32–69 (44)
	1,000,000	4/4	48–69 (50)
LAPC9-$\alpha2\beta1^{+/hi}$	100	0/4	
	1,000	0/4	
	10,000	1/4	109
LAPC9-$\alpha2\beta1^{-/lo}$	100	0/4	
	1,000	0/4	
	10,000	1/4	109
	100,000	4/4	42–102 (48)

*Tumor cells were injected in Matrigel into the dorsal prostate of NOD/SCID mice. Tumor incidence refers to the number of tumors developed/number of injections.

† Tumor latency refers to the time (in days) from tumor cell injection to when the tumor is detected by palpation. The numbers in parentheses represent the median values.

development, and 500,000 cells were required to reliably generate tumors in a similar time frame (Table 1). LAPC9 cells, on the other hand, were considerably more tumorigenic because 10,000 cells could initiate tumors with 50% efficiency in about the same time interval required for 500,000 Du145 or LAPC4 cells to form tumors (Table 1). When tumorigenicity assays were done using the purified isogenic $\alpha2\beta1^{+/hi}$ and $\alpha2\beta1^{-/lo}$ populations in LAPC4 tumors, again, we did not observe an enrichment in tumor-initiating cells in the $\alpha2\beta1^{+/hi}$ subset (Table 1). Rather, the $\alpha2\beta1^{-/lo}$ population seemed slightly more tumorigenic because tumor formation was observed when 10,000 $\alpha2\beta1^{-/lo}$ cells were injected whereas no tumors formed when 10,000 $\alpha2\beta1^{+/hi}$ or unsorted cells were injected (Table 1). Similar experiments were carried out using sorted LAPC9 cells and, in this case, unsorted, $\alpha2\beta1^{+/hi}$, and $\alpha2\beta1^{-/lo}$ cells showed very similar tumorigenicity (Table 1).

Due to the overall low tumor development of human prostate cancer cells implanted into the dorsal prostate of NOD/SCID mice, we carried out tumor experiments by injecting cells s.c. as we have recently shown that the subcutis is very permissive to tumor regeneration relative to the mouse dorsal prostate.[3] Indeed, as few as 100 LAPC9 cells injected s.c. initiated 50% tumor development (Supplementary Table S2) compared with 10,000 cells required to initiate similar levels of tumor development when injected into the dorsal prostate (Table 1). One thousand s.c. injected LAPC9 cells initiated tumor development in 100% recipient animals in ~2 months (Supplementary Table S2). Nevertheless, despite the dramatically enhanced tumor take in the s.c. implantation model, purified $\alpha2\beta1^{+/hi}$ and $\alpha2\beta1^{-/lo}$ LAPC9 cells did not show a significant difference with respect to their tumor-initiating capacities (Supplementary Table S2). If anything, the $\alpha2\beta1^{-/lo}$ LAPC9 cells seemed to be a bit more tumorigenic than the isogenic $\alpha2\beta1^{+/hi}$ cells because the latter cells, at lower cell numbers (i.e., 100 and 1,000 cells), regenerated more tumors (Supplementary Table S2).

The preceding experiments (Fig. 2; Table 1; Supplementary Table S2) reveal that the small population of $\alpha2\beta1^{+/hi}$ prostate cancer cells have high clonal and clonogenic capacities *in vitro* but are no more tumorigenic *in vivo* than the corresponding $\alpha2\beta1^{-/lo}$ prostate cancer cells. The behavior of $\alpha2\beta1^{+/hi}$ cells is reminiscent of that of the $ABCG2^+$ cells (23) and suggests that the $\alpha2\beta1^{+/hi}$ prostate cancer cells, like $ABCG2^+$ cells, may mark a population of fast-proliferating tumor progenitor cells. This would imply that, like the $ABCG2^-$ cell population (23), the $\alpha2\beta1^{-/lo}$ prostate cancer cell population might contain primitive tumorigenic cells that can give rise to $\alpha2\beta1^{+/hi}$ cells. In support, tumors derived from 100 highly purified $\alpha2\beta1^{-/lo}$ Du145 cells contained a small percentage of $\alpha2\beta1^{+/hi}$ cells (Supplementary Table S1), suggesting that some $\alpha2\beta1^{-/lo}$ cells have generated $\alpha2\beta1^{+/hi}$ cells. We further analyzed several s.c. or surgical orthotopic implantation tumors derived from 1,000 to 100,000 $\alpha2\beta1^{-/lo}$ LAPC9 cells and, in every case, $\alpha2\beta1^{+/hi}$ cells were present at a low frequency (0.1–3.9%; Supplementary Table S1). Likewise, surgical orthotopic implantation tumors arising from 100,000 $\alpha2\beta1^{-/lo}$ LAPC4 and Du145 cells also contained a consistent small number of $\alpha2\beta1^{+/hi}$ cells (Supplementary Table S1). These results suggest that some $\alpha2\beta1^{-/lo}$ cells can give rise to $\alpha2\beta1^{+/hi}$ cells *in vivo*.

CD44, α2β1, and ABCG2 identify overlapping and inclusive prostate cancer cell populations. We have recently shown that the $CD44^+$ cell population is enriched in prostate cancer tumor stem/progenitor cells (24) whereas ABCG2 identifies fast-cycling tumor progenitor cells (23). The preceding experiments suggest that α2β1 may also mark fast-cycling tumor progenitor cells. Next, we carried out immunostaining in an attempt to elucidate the interrelationship among the three (i.e., $CD44^+$, $\alpha2\beta1^{+/hi}$, and $ABCG2^+$) cell populations. We sorted out $\alpha2\beta1^{+/hi}$ and $\alpha2\beta1^{-/lo}$ cells from Du145 xenograft tumors, plated them onto coverslips immediately after sorting, and fixed them 3 h later to prevent them from dividing. When double-stained for CD44, we found that there was a significant overlap between the $\alpha2\beta1^{+/hi}$ and

[3] H.W. Li, M. Jiang, T. Calhoun-Davis, L. Patrawala, G. Choy, R. Schneider-Broussard, S.W. Hayward, D.G. Tang. Crucial roles of microenvironments (transplantation sites) on reconstituting tumorigenic versus metastatic potentials of human prostate cancer (stem) cells in nonobese diabetic/severe combined immunodeficient mice. Submitted for publication.

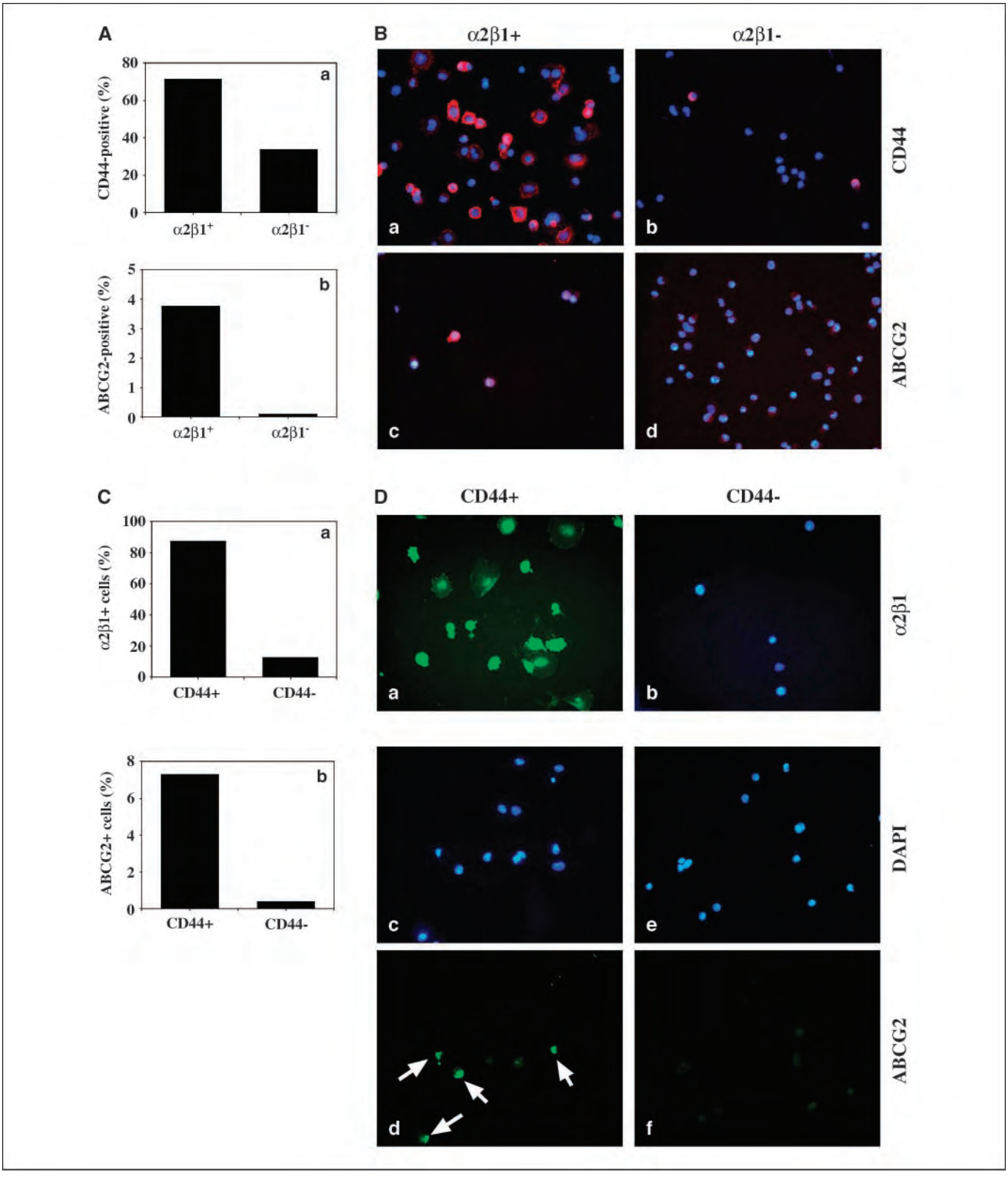

Figure 3. CD44, α2β1, and ABCG2 identify overlapping prostate cancer cell populations. *A* and *B,* CD44 and ABCG2 expression in $\alpha2\beta1^+$ and $\alpha2\beta1^-$ Du145 cells. Purified $\alpha2\beta1^+$ and $\alpha2\beta1^-$ Du145 cells were plated at 5,000 per 18-mm^2 coverslip and fixed after 4 h. *B,* cells were stained using indirect fluorescence with antibodies against CD44 (*a* and *b*) or ABCG2 (*c* and *d*). *A,* the majority of the $\alpha2\beta1^+$ were also positive for CD44 (*a*). ABCG2 was almost exclusively expressed in the $\alpha2\beta1^+$ population (*b*). *C* and *D,* α2β1 and ABCG2 expression in $CD44^+$ and $CD44^-$ Du145 cells. Purified $CD44^+$ and $CD44^-$ Du145 cells were plated at 5,000 per 18-mm^2 coverslip and fixed after 4 h. *D,* cells were stained using indirect fluorescence with antibodies against α2β1 (*a* and *b*) or ABCG2 (*c* and *f*). *C,* consistent with (*A*) and (*B*), the majority of the $CD44^+$ cells were also positive for α2β1 (*a*). The $ABCG2^+$ cell population also localized mostly to the $CD44^+$ population (*b*). *D, d, arrowheads,* $ABCG2^+$ cells.

$CD44^+$ populations [i.e., ~70% of the $\alpha2\beta1^{+/hi}$ cells were also $CD44^+$ (by prediction, ~30% of the $\alpha2\beta1^{+/hi}$ cells were $CD44^-$) and ~30% $\alpha2\beta1^{-/lo}$ cells were $CD44^+$ (Fig. 3*A,a* and *B,a–b*)]. When double-stained for ABCG2, which normally is expressed in ~1% to 5% of the total population (23), we found that nearly all $ABCG2^+$ cells lay within the $\alpha2\beta1^{+/hi}$ subset (Fig. 3*A,b* and *B,c–d*).

We then carried out the reciprocal experiment (i.e., we isolated $CD44^+$ and $CD44^-$ Du145 cells from xenograft tumors and stained them for $\alpha2\beta1$ and ABCG2; Fig. 3*C* and *D*). As expected, we observed that ~80% of $CD44^+$ cells also expressed $\alpha2\beta1$ (i.e., $CD44^+\alpha2\beta1^+$) and that essentially all the $ABCG2^+$ cells were localized in the $CD44^+$ cell population. These results suggest that CD44, $\alpha2\beta1$, and ABCG2 mark overlapping populations of prostate cancer cells with differing tumorigenic properties.

Tumorigenic hierarchy revealed by CD44 and α2β1 expression profiles. The above experiments suggest that prostate cancer cells can be stratified, according to their CD44 and $\alpha2\beta1$ expression profiles, to at least four different cell populations (i.e., $CD44^+\alpha2\beta1^+$, $CD44^+\alpha2\beta1^-$, $CD44^-\alpha2\beta1^+$, and $CD44^-\alpha2\beta1^-$). We used the LAPC9 xenograft tumor model to address whether these populations of cells have intrinsic differences with respect to their tumorigenicity. In LAPC9 tumors, these four populations of cells represent 0.28 ± 0.2%, 9 ± 4.7%, 1.4 ± 0.8%, and 91 ± 5.3% (n = 5), respectively (Fig. 4*A*), suggesting that the bulk of the tumor cell population is $CD44^-\alpha2\beta1^{-/lo}$ and the single-positive and double-positive cells represent the minority. Because we have previously shown that most $CD44^+$ LAPC9 cells are androgen receptor negative and essentially all androgen receptor–positive cells are localized in the $CD44^-$ cell population (24), the above results (Fig. 4*A*) suggest that the bulk of the LAPC9 tumors may be differentiated cells. Indeed, immunostaining revealed that most tumor cells were androgen receptor positive (not shown). When 1,000 LAPC9 cells of each phenotype were injected s.c. into male NOD/SCID mice supplemented with testosterone, the $CD44^+\alpha2\beta1^+$ and $CD44^+\alpha2\beta1^-$ cells showed similar tumor take (i.e., 100%) and tumor growth (Table 2; Fig. 4*B*). The $CD44^-\alpha2\beta1^+$ LAPC9 cells showed slightly lower tumor take (i.e., 90%) and initiated smaller tumors (P = 0.022; Table 2; Fig. 4*B*). The $CD44^-\alpha2\beta1^-$ cells showed the lowest tumor incidence (i.e., 40%) and tumors were significantly smaller than the $CD44^+\alpha2\beta1^+$ or $CD44^+\alpha2\beta1^-$ cell-initiated tumors (Table 2; Fig. 4*B*). When 10,000 highly purified LAPC9 cells of each phenotype were used in the same set of experiments, similar patterns in tumorigenicity were observed although tumors initiated by $CD44^+\alpha2\beta1^-$ and $CD44^-\alpha2\beta1^+$ cells were of similar sizes (Table 2; Fig. 4*C*). When 10,000 acutely purified $CD44^+\alpha2\beta1^+$ or $CD44^-\alpha2\beta1^-$ LAPC9 cells were injected s.c. into the male NOD/SCID mice without exogenous testosterone, the $CD44^+\alpha2\beta1^+$ cells generated more tumors, which were nearly 20 times bigger

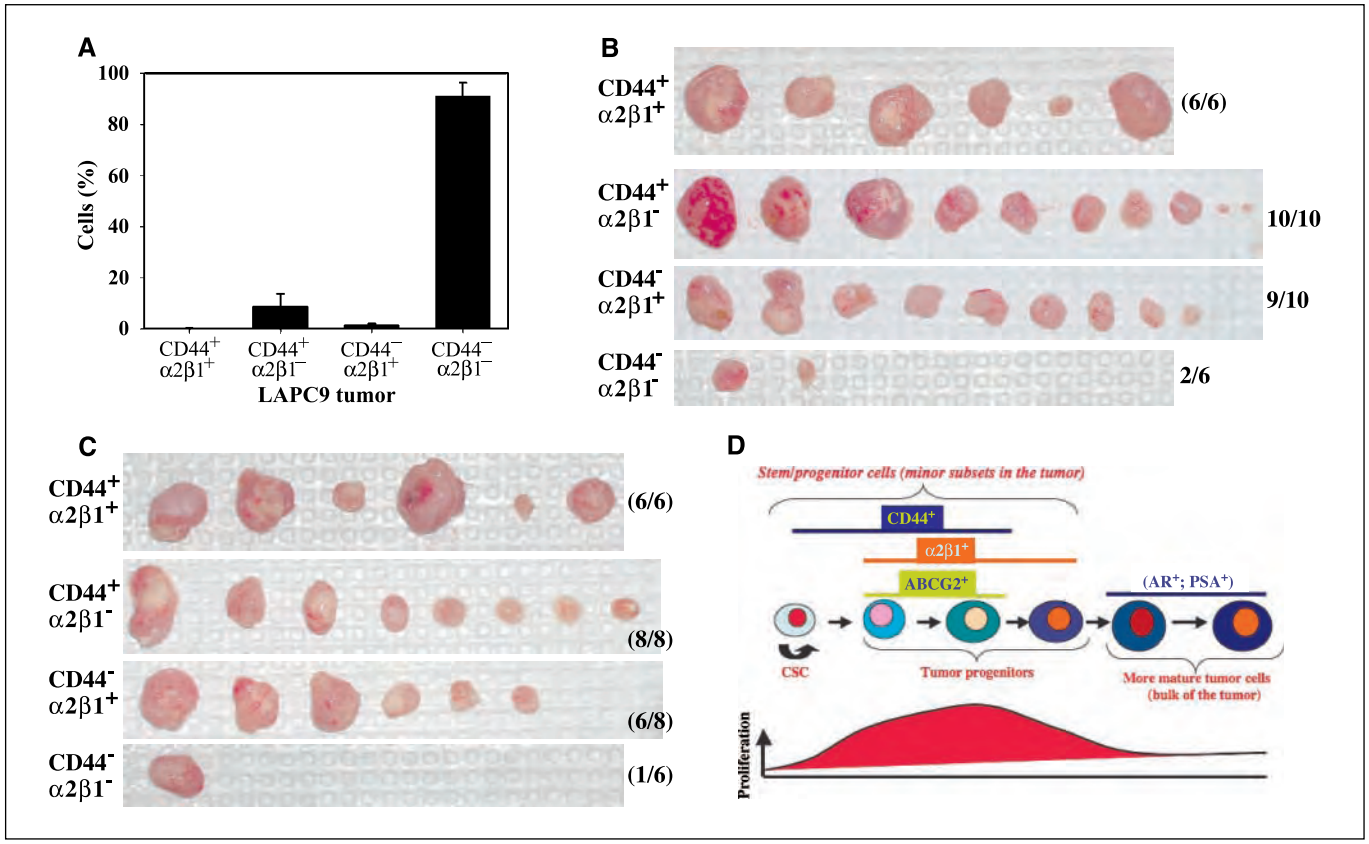

Figure 4. Hierarchical organization of tumor cells in LAPC9 xenograft tumors. *A,* the percentages of tumor cells of different phenotypes in LAPC9 xenograft tumors analyzed by flow cytometry analysis. *Columns,* mean (n = 5); *bars,* SD. *B* and *C,* tumor experiments. One thousand (*B*) or 10,000 (*C*) acutely purified LAPC9 cells of four different phenotypes were injected s.c. in Matrigel into male NOD/SCID mice supplemented with exogenous testosterone pellets. Tumor images and incidences were indicated. See Table 2 for more details. *D,* a hypothetical model of hierarchical organization of prostate cancer cells. Prostate tumors contain the bulk differentiating and differentiated mature tumor cells that express CD57, androgen receptor (*AR*), and prostate-specific antigen (*PSA*), as well as small populations of undifferentiated tumorigenic cells that can be identified by CD44 expression. The $CD44^+$ cell population encompasses both tumor progenitors that are $ABCG2^+$ and $\alpha2\beta1^+$ and are highly proliferative (*bottom*) and slow-cycling cancer stem cells (*CSC*) that seem to be $ABCG2^-$ and $\alpha2\beta1^-$. Cancer stem cells are hypothesized to have the ability to self-renew. See text for more details.

Table 2. Tumorigenicity of s.c implanted double-sorted LAPC9 cells

Phenotype	No. cells	Incidence (%)*	Termination (d) †	Weight (g) ‡	P§
With dihydrotestosterone					
$CD44^+\alpha2\beta1^+$	1,000	6/6 (100)	60	0.43 ± 0.30 (0.02–0.72)	
$CD44^+\alpha2\beta11^-$	1,000	10/10 (100)	60	0.45 ± 0.57 (0.09–1.76)	0.63
$CD44^-\alpha2\beta11^+$	1,000	9/10 (90)	60	0.19 ± 0.17 (0.05–0.48)‖	0.022
$CD44^-\alpha2\beta11^-$	1,000	2/5 (40)¶	60	0.03 ± 0.05 (0.03–0.11)**	0.017
$CD44^+\alpha2\beta11^+$	10,000	6/6 (100)	60	0.48 ± 0.39 (0.16–1.1)	
$CD44^+\alpha2\beta11^-$	10,000	8/8 (100)	60	0.18 ± 0.21 (0.02–0.65)	0.09
$CD44^-\alpha2\beta11^+$	10,000	6/8 (75)	60	0.28 ± 0.25 (0.04–0.60)	0.15
$\alpha2\beta11^-CD44^-$	10,000	1/6 (17)¶	60	0.3	0.029
No dihydrotestosterone					
$\alpha2\beta11^+/CD44^+$	10,000	5/6 (83)	55	1.46 ± 0.5 (0.55–1.1)	
$\alpha2\beta11^{-/}CD44^-$	10,000	3/6 (50)¶	55	0.08 ± 0.04 (0.03–0.11)	0.035

*Tumor cells were injected in Matrigel s.c. into male NOD/SCID mice supplemented with or without testosterone pellets. Tumor incidence (indicated as percent in the parentheses) refers to the number of tumors developed/number of injections.
†Time (in days) when animals were terminated.
‡Mean ± SD. Tumor weight range is indicated in parentheses.
§Unpaired Student's *t* test (all compared with double positive cells).
‖$P = 0.19$, compared with $CD44^+\alpha2\beta11^-$.
¶$P < 0.05$, compared with double-positive cells.
**$P = 0.05$, compared with $CD44^+\alpha2\beta11^-$.

than the tumors initiated by corresponding $CD44^-\alpha2\beta1^-$ cells (Table 2). Interestingly, tumors initiated by 10,000 $CD44^+\alpha2\beta1^+$ cells in male NOD/SCID mice without exogenous testosterone (Table 2, *bottom*) were about thrice larger than the tumors initiated by the same number of $CD44^+\alpha2\beta1^+$ cells in mice with exogenous testosterone (Table 2, *top*). Similar differences in tumorigenicity between the double-positive and double-negative LAPC9 cell populations were also observed when 10,000 $CD44^+\alpha2\beta1^+$ or $CD44^-\alpha2\beta1^-$ cells were implanted in female NOD/SCID mice, with average tumor weights being 1.62 and 0.029 g ($P < 0.001$), respectively.

Discussion

It has long been known that human tumors, although clonal by origin, are rather heterogeneous in their cellular composition. The cancer stem cell hypothesis helps explain this biological conundrum (1). Recent studies in breast and colon cancers as well as gliomas suggest that tumor cells *in vivo* may indeed be organized as a hierarchy with tumor-initiating cells or cancer stem cells sitting at the apex and having the ability to develop (or differentiate) into a spectrum of more mature progeny (see Introduction). Our work (9, 23, 24) and the work of Collin et al. (22) suggest that human prostate cancer cells may also be organized as a hierarchy. The present study provides concrete experimental evidence for a hierarchical organization of tumor cells in xenograft human prostate tumors.

Our previous studies have revealed that in several xenograft human tumors, the $CD44^+$ cell population is enriched in tumorigenic prostate cancer stem and progenitor cells (9, 24). When highly purified cells are used in surgical orthotopic implantation experiments, most tumorigenicity and all metastatic ability are localized in the $CD44^+$ population (24). Serial sphere-formation assays, label-retaining experiments, "stemness" gene expression profiling, clonal analyses, and asymmetrical segregation of CD44 indicate that the $CD44^+$ prostate cancer cell population is still heterogeneous, with only ~1% of the cells in this population representing cancer stem cells and the majority representing highly proliferative tumor progenitors (9, 24). These observations suggest that the $CD44^+$ prostate cancer cell population is still heterogeneous consisting of perhaps subsets of cells with differing tumor-initiating abilities (hence the name $CD44^+$ prostate cancer stem/progenitor cells; ref. 9). This suggestion is consistent with studies showing that the leukemic stem cells in acute myelogenous leukemia (AML) identified by $CD34^+CD38^-$ (29) and colon cancer–initiating cells (or colon cancer stem cell) identified by CD133 (6) are heterogeneous populations of cells with true cancer stem cells representing only a minor subset (i.e., 0.1–1%).

Bearing in mind that the $CD44^+$ prostate cancer cell population is heterogeneous, we seek to further dissect tumorigenic prostate cancer cell subsets in the current study. We put our focus on $\alpha2\beta1^{+/hi}$ prostate cancer cells because this integrin receptor has been reported to mark a population of normal human prostate stem cells (21). Of great interest, highly purified $\alpha2\beta1^{+/hi}$ prostate cancer cells *in vitro* possess higher cloning and clonogenic potentials than the corresponding $\alpha2\beta1^{-/lo}$ cells. However, when put *in vivo*, either orthotopically in dorsal prostate or s.c., the $\alpha2\beta1^{+/hi}$ cells exhibit very similar tumorigenicity to $\alpha2\beta1^{-/lo}$ cells. The different behavior of the $\alpha2\beta1^{+/hi}$ prostate cancer cells *in vitro* and *in vivo* is in sharp contrast to the $CD44^+$ prostate cancer cells (ref. 24, and this study) and highlights the critical importance of carrying out tumor experiments when assaying candidate cancer stem cell populations because cells showing enhanced *in vitro* clonal capacity and clonogenic potential may not necessarily represent primitive tumor-initiating cells (and therefore should not be called cancer stem cells because tumor initiation is the "gold" standard in defining cancer stem cell; refs. 8, 9).

On the other hand, the $\alpha2\beta1^{+/hi}$ prostate cancer cells behave very similarly to the $ABCG2^+$ cancer cells, which represent

fast-proliferating tumor progenitors (23). Indeed, both ABCG2$^+$ and $\alpha2\beta1^{+/hi}$ prostate cancer cells are highly proliferative and clonogenic *in vitro* but are not more tumorigenic than the corresponding marker negative cell populations (ref. 23, and this study). Double immunofluorescence staining experiments reveal that the ABCG2$^+$ cells are all localized in the α2β1- and CD44-expressing cell populations and that ~70% of the $\alpha2\beta1^+$ prostate cancer cells are also positive for CD44. Using dual fluorescence sorting, tumor cells in LAPC9 xenografts are fractionated into the bulk (i.e., >90%) $CD44^-\alpha2\beta1^-$ cells and minor subsets of single-positive or double-positive cells. Remarkably, although constituting <10% of the total tumor cell population, the $CD44^+\alpha2\beta1^+$ as well as $CD44^+\alpha2\beta1^-$ cells, on an equal cell number basis, show much higher tumor-initiating abilities than the $CD44^-\alpha2\beta1^-$ cells. These observations, together with our findings that most CD44$^+$ prostate cancer cells are androgen receptor negative (24), lead us to propose a hypothetical model for the hierarchical organization of human prostate cancer cells (Fig. 4*D*). The bulk of the tumor cells in prostate cancer is differentiating and differentiated cells expressing androgen receptor and prostate-specific antigen. These mature cells possess low tumor-initiating activity whereas most tumorigenicity resides in the minor CD44$^+$ cell population (Fig. 4*D*; ref. 24).

Several pieces of evidence provide support for this model. First, most tumorigenicity resides in the relatively small population of CD44$^+$ cells, which range from ~1% to 20% in xenograft tumors (ref. 24, and this study). In primary patient tumors, interestingly, the percentage of CD44$^+$ cells seems to correlate with the Gleason grade, with Gleason grade 6 to 9 tumors having ~3%, 9%, 18%, and 19% of CD44$^+$ prostate cancer cells.[4] Second, the CD44$^+$ prostate cancer cell population is still heterogeneous, encompassing tumor progenitor cells that are $ABCG2^+\alpha2\beta1^+$ and relatively quiescent, slow-cycling cancer stem cells that are $CD44^+ABCG2^-\alpha2\beta1^-$ (Fig. 4*D*; ref. 24, and this study). In support of this conjecture, all ABCG2$^+$ cells and most (i.e., 70–80%) of the $\alpha2\beta1^+$ cells are included in the CD44$^+$ cell population, and overall, the $CD44^+\alpha2\beta1^+$ and $CD44^+\alpha2\beta1^-$ LAPC9 cells have very similar tumorigenicities. In fact, the tumorigenicity of CD44$^+$ (i.e., sorted using a single marker) cells is also indistinguishable from that of $CD44^+\alpha2\beta1^+$ or $CD44^+\alpha2\beta1^-$ cells (ref. 24, and this study), suggesting that fluorescence-activated cell sorting (FACS) using either CD44 alone or CD44/α2β1 combination is purifying practically the same prostate cancer cell population. Primary human tumors also reveal that ~75% of the $\alpha2\beta1^+$ cells are localized in the CD44$^+$ prostate cancer cell population. Third, the $\alpha2\beta1^+$ and $\alpha2\beta1^-$ cells are not significantly different in terms of their tumorigenicity, which can be explained by the fact that ~30% of the CD44$^+$ cells are localized in the $\alpha2\beta1^-$ cell population (Fig. 3). In fact, the $\alpha2\beta1^-$ population seems to be slightly enriched in tumorigenic cells. For example, 100,000 $\alpha2\beta1^-$ Du145 cells orthotopically implanted in the dorsal prostate can initiate tumor in four of the four injections, whereas the same number of unfractionated Du145 cells cannot initiate any tumor development (Table 1). In addition, all tumors derived from the $\alpha2\beta1^-$ cells contain small numbers of $\alpha2\beta1^+$ cells (see Supplementary Table S1). Remarkably, in tumors derived from high numbers (i.e., 100,000) of the $\alpha2\beta1^-$ LAPC4 or LAPC9 cells, more $\alpha2\beta1^+$ cells are observed than in unsorted tumors (Supplementary Table S1). All these observations support the hypothesis that $\alpha2\beta1^-$ population contains more primitive cells that can "regenerate" $\alpha2\beta1^+$ cells. Furthermore, when injected s.c., 100 $\alpha2\beta1^-$ LAPC9 cells, like the unsorted cells, can initiate 50% tumor development whereas 10 times more $\alpha2\beta1^+$ cells are required to achieve similar tumor take. These data suggest that ~30% of the CD44$^+$ prostate cancer cells that are $\alpha2\beta1^-$ might harbor primitive self-renewing cancer stem cells (Fig. 4*D*). Fourth, the $CD44^+\alpha2\beta1^-$ cells and $CD44^-\alpha2\beta1^+$ cells behave very similarly, in terms of their tumor-initiating abilities, to the $\alpha2\beta1^-$ and $\alpha2\beta1^+$ cells, respectively. In addition, we have previously shown that 1,000 $CD44^-$ LAPC9 cells injected s.c. can initiate tumor development in five of the six injections (24), suggesting that there exist tumorigenic cells in the $CD44^-$ cell population. In the present study, we find that 1,000 highly purified $CD44^-\alpha2\beta1^+$ cells initiate tumor development in 9 of the 10 implantations (Fig. 4*B*), suggesting that tumorigenic cells in $CD44^-$ population might all be $\alpha2\beta1^+$ (i.e., having the $CD44^-\alpha2\beta1^+$ phenotype). These results emphasize the important concept that tumor progenitor cells, like the putative primitive cancer stem cells, can be tumorigenic in regular tumor assays. Presumably, exhaustive serial tumor transplantation experiments can functionally distinguish putative cancer stem cells from tumor progenitors (Fig. 4*D*; ref. 9). Finally, in all xenograft models (DNp53-T, Du145, LAPC4, and LAPC9) as well as primary patient samples we have studied, the percent of CD44$^+$ cells is always higher than that of $\alpha2\beta1$ (24),[5] supporting that CD44 marks both cancer stem cells and tumor progenitors whereas $\alpha2\beta1$ expression identifies a subset of tumor progenitors (Fig. 4*D*).

The most remarkable finding in this study is that the CD44$^+$ cell population (including both $CD44^+\alpha2\beta1^+$ and $CD44^+\alpha2\beta1^-$ cells) has much higher tumorigenicity than $CD44^-\alpha2\beta1^-$ cells. Intriguingly, when the $CD44^+\alpha2\beta1^+$ cells are implanted in either male NOD/SCID mice without exogenous testosterone or in female NOD/SCID mice, tumors developed are more than thrice larger than when the double-positive cells are implanted in male NOD/SCID mice supplemented with exogenous testosterone pellets. Because the former two experimental settings are likely androgen deficient, it is tempting to speculate that under these conditions, the cancer stem cell–containing $CD44^+\alpha2\beta1^+$ cell population, mostly androgen receptor negative (24), may preferentially proliferate, leading to higher tumor growth.

An obvious question pertains to the phenotypic properties of the putative cancer stem cells in the CD44$^+$ prostate cancer cell population (Fig. 4*D*). The CD133$^+$ cells may represent good candidates because they have been reported to mark normal prostate stem cells (21) and potential prostate cancer stem cells with higher clonogenic potential (although tumorigenic potential has not been studied; ref. 22). We have also found that primary patient tumor samples contain 0.25% to 1.4% CD133$^+$ cells and that the CD133$^+$ prostate cancer cells purified from LAPC4 xenograft and HPCa13 patient tumors possess higher clonal and clonogenic potentials.[4] Studies are under way to characterize the *in vivo* tumorigenicity of $CD44^+CD133^+$ prostate cancer cells and to determine whether they may represent human prostate cancer stem cells. Of particular interest, CD133 has recently been used as a marker to prospectively identify brain and colon tumor–initiating

[4] L. Patrawala and D.G. Tang, unpublished observations.

[5] This study; unpublished observations.

cells (5–7), suggesting that this surface molecule, whose biological functions are yet to be elucidated, may represent more or less a "universal" normal stem cell and cancer stem cell marker. Another potential candidate population of primitive prostate cancer stem cells might be in side population, which, in LAPC9 tumors, represents ~0.1% and has even higher tumorigenicity than the $CD44^+$ cells (23, 24).

Regardless, our work (ref. 24, and this study) provides concrete experimental rationale for using CD44 as a marker to identify tumorigenic prostate cancer cells. This rationale is also in line with others' studies using CD44 as the positive surface marker to identify tumor-initiating cells in breast (4), head and neck (30), and pancreatic (31) cancers. Our findings that the $CD44^+$ prostate cancer cell population likely contains both cancer stem cells and tumor progenitors (ref. 24, and this study) and that the $CD44^-\alpha 2\beta 1^-$ cells, which constitute the bulk of the tumor, are much less tumorigenic (Fig. 4*D*) suggest that targeting $CD44^+$ prostate cancer cell population may represent a viable approach to prostate cancer therapy. This therapeutic strategy is supported by the recent finding that CD44 is required for leukemic stem cells to engraft in the bone marrow (32, 33) and strongly encouraged by the success of using anti-CD44 antibody to target AML stem cells and nearly cure the disease in mice (33). We are currently designing experimental therapeutics to specifically target the $CD44^+$ prostate cancer cells.

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table S1. $\alpha2\beta1^{+/hi}$ and $\alpha2\beta1^{-/lo}$ cells in xenograft prostate tumors

Cells derived from[a]	$\alpha2\beta1^{+}$ cells (%)[b]	n
LAPC-9		
Unsorted s.c tumor	2.4 ± 2.3	9
1 k $\alpha2\beta1^{+/hi}$ s.c tumor	undetectable	2
10 k $\alpha2\beta1^{+/hi}$ s.c tumor	0.1	1
100 $\alpha2\beta1^{-/lo}$ s.c tumor	2.1 ± 2.8	2
1 k $\alpha2\beta1^{-/lo}$ s.c tumor	1.2 ± 0.2	2
10 k $\alpha2\beta1^{-/lo}$ s.c tumor	0.4 ± 0.3	2
100 k $\alpha2\beta1^{-/lo}$ s.c tumor	3.9 ± 3.3	2
10 k $\alpha2\beta1^{+/hi}$ SOI tumor	2.1	1
10 k $\alpha2\beta1^{-/lo}$ SOI tumor	0.1	1
LAPC-4		
Unsorted s.c tumor	0.4 ± 0.5	5
100 k $\alpha2\beta1^{-/lo}$ SOI tumor	5.1 ± 4.0	3
Du145		
Unsorted s.c. tumor	9.8 ± 2.7	4
100 k $\alpha2\beta1^{-/lo}$ SOI tumor	8.1 ± 3.0	2

[a]Xenograft tumors derived from either unsorted or sorted cells injected either subcutaneously (s.c) or orthotopically into the dorsal prostate (i.e., surgical orthotopic implantation or SOI) were harvested to prepare single-cell human tumor cell suspension (see Materials and methods), which was then used in α2β1 staining followed by fluorescence microscopy and/or flow cytometry analysis.

[b]Mean ± S.D.

table S2. Tumorigenicity of *s.c* implanted $\alpha2\beta1^{+/hi}$ and $\alpha2\beta1^{-/lo}$ LAPC9 cells

LAPC9 cells	Cell#	Incidence[a]	Latency (days)[b]
Unsorted	100	3/6	96-124 (96)
	1,000	6/6	60-67 (64)
$\alpha2\beta1^{+/hi}$	100	0/6	
	1,000	3/6	66 -82 (74)
	10,000	5/6	60 -82 (74)
$\alpha2\beta1^{-/lo}$	100	3/6	75-96 (89)
	1,000	6/8	68-89 (68)
	10,000	5/8	56-75 (56)
	100,000	4/6	40-40 (40)

[a]Tumor cells were injected in Matrigel subcutaneously (s.c) into the NOD/SCID mice. Tumor incidence refers to the number of tumors developed/number of injections.

[b]Tumor latency refers to the time (in days) from tumor cell injection to when the tumor is detected by palpation. The numbers in parentheses represent the median values.